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(54) Expression of human serum albumin in *pichia pastoris*.

(57) A novel expression cassette, vectors and process for the secretion of HSA in *Pichia pastoris* cells. In accordance with the present invention there has been discovered an improved expression cassette for the production of HSA in *Pichia pastoris* comprising

- a *Pichia pastoris* 5' regulatory region having a 5' end and a 3' end selected from the group consisting of the *Pichia pastoris* AOX1 5' regulatory region and the *Pichia pastoris* DAS1 5' regulatory region wherein the 3' end of the 5' regulatory region is operably linked to;
- an HSA structural gene encoding a signal sequence and mature protein having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within about 11 deoxyribonucleotides of the 5' end of said HSA structural gene; and operably linked to
- a 3' termination sequence.

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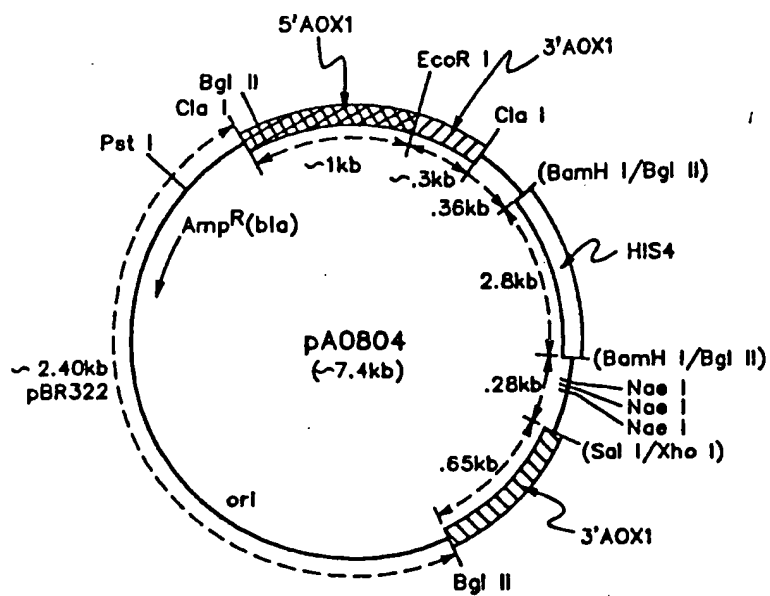


FIG. 1

Field of the Invention

This invention relates to the field of recombinant DNA biotechnology. In one aspect, this invention relates to a process for the expression of human serum albumin (HSA) in *Pichia pastoris*.

Background

Human serum albumin is the most abundant plasma protein of adults. The concentration of albumin is 40 mg/ml, or 160g of albumin circulating throughout the human body for a 70 kg adult male. This protein maintains osmotic pressure and functions in the binding and transport of copper, nickel, calcium (weakly, at 2-3 binding sites), bilirubin and protoporphyrin, long-chain fatty acids, prostaglandins, steroid hormones (weak binding with these hormones promotes their transfer across the membranes), thyroxine, triiodothyronine, and glutathione. According to Peters, T. and Reed, R.G. in Albumin: Structure, Biosynthesis and Function, (Peters, T. and Sjöholm, J. eds.) 1977 p.11-20, over 10,000 kilograms of purified albumin are administered annually in the United States alone to patients with circulatory failure or with albumin depletion.

Currently the only commercial source of HSA is from fractionated blood. Considering the possible dangers of blood borne contaminants and pathogens, it would be a considerable contribution to the commercial production of HSA to develop alternate methods of producing HSA. With the advent of recombinant DNA technology, it is now possible to produce HSA by alternate methods.

HSA has also been expressed in *Saccharomyces cerevisiae* as disclosed by Etcheverry et al. in Bio/technology, August 1986, p. 726 and Arjum Singh in EPA 123,544. Etcheverry disclosed HSA expression intracellularly in a concentration of approximately 6 mg/l and the presence of cell-associated HSA. Hayasuke et al. also disclosed the expression of HSA in *Saccharomyces cerevisiae* in combination with the GAL 1 promoter and a signal sequence. Hayasuke et al. appears to have been able to achieve a secreted production level of 160 mg/l. As described in EPA344,459, HSA has also been expressed in *Pichia pastoris* as intracellular or cell-associated protein. Although the expression of HSA in yeast cells, such as *Saccharomyces cerevisiae* and *Pichia pastoris*, is a significant step toward providing safe alternative sources of HSA, the expression of HSA as an intracellular or cell-associated protein is not desirable. Expensive and time consuming measures are required to recover and purify intracellular or cell-associated HSA. Furthermore, it would also be advantageous if a high level of HSA secretion could be achieved to improve the yields and lower the production costs.

Thus, it would be a significant contribution to the art to provide yeast strains which secrete high levels of HSA.

It also would be a significant contribution to the art to develop a process which produces HSA in a manner that is easy to recover and purify.

Therefore, it is an object of this invention to provide strains which produce high levels of HSA.

It is a further object of this invention to provide a process which produces HSA in a manner that is easy to recover and purify.

Other objects and advantages of the present invention will be apparent to those skilled in the art from the present specification.

Summary of the Invention

In accordance with the present invention there has been discovered an improved expression cassette for the production of HSA in *Pichia pastoris* comprising

- a) a 5' *Pichia pastoris* regulatory region having a 5' end and a 3' end selected from the group consisting of the *Pichia pastoris* AOX1 regulatory region and the *Pichia pastoris* DAS1 regulatory region wherein the 3' end of the regulatory region is operably linked to
- b) an HSA structural gene encoding a signal sequence and mature protein having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within about 6 deoxyribonucleotides of the 5' end of said HSA structural gene; and operably linked to
- c) a 3' termination sequence.

In accordance with the present invention there has also been discovered *Pichia pastoris* strains transformed with an improved expression cassette for the production of HSA in *Pichia pastoris* comprising

- a) a 5' *Pichia pastoris* regulatory region having a 5' end and a 3' end selected from the group consisting of the *Pichia pastoris* AOX1 regulatory region and the *Pichia pastoris* DAS1 regulatory region wherein the 3' end of the regulatory region is operably linked to

b) an HSA structural gene encoding a signal sequence and mature protein having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within about 6 deoxyribonucleotides of the 5' end of said HSA structural gene; and operably linked to
c) a 3' termination sequence.

In a further embodiment of the present invention, there has also been discovered a process for the secretion of HSA from transformed *Pichia pastoris* cells comprising

a) transforming *Pichia pastoris* with at least one vector having at least one expression cassette comprising

i) a 5' *Pichia pastoris* regulatory region having a 5' end and a 3' end selected from the group consisting of the *Pichia pastoris* AOX1 regulatory region and the *Pichia pastoris* DAS1 regulatory region wherein the 3' end of the regulatory region is operably linked to

ii) an HSA structural gene encoding a signal sequence and mature protein having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within about 6 deoxyribonucleotides of the 5' end of said HSA structural gene; and operably linked to

iii) a 3' termination sequence; and then

b) culturing the resulting transformed *Pichia pastoris* under suitable conditions to obtain the secretion of HSA.

Detailed Description of the Figures

Figure 1 provides a representation of plasmid pA0804 which contains a linear site-specific integrative vector in the fragment clockwise from BglII to BglII. The structural gene may be inserted in the unique EcoRI site of this plasmid. This plasmid may be recovered from the plasmid DNA of NRRL B-18114 by EcoRI digest and gel electrophoresis to recover a linear ~7.4 kb EcoRI fragment corresponding to Figure 1.

Figure 2 provides a representation of pHSA13 in circular form.

Figure 3 provides a restriction map of the AOX1 5' regulatory region isolated from *Pichia pastoris*.

Figure 4 provides a restriction map of the DAS1 5' regulatory region isolated from *Pichia pastoris*.

Figure 5 provides a restriction map of the AOX1 3' termination sequence isolated from *Pichia pastoris*.

Figure 6 provides a restriction map of the DAS1 3' termination sequence isolated from *Pichia pastoris*.

Figure 7 provides a representation of pHSA113 in linear form.

Figure 8 provides a representation of plasmid pA0807N which contains a linear site-specific integrative vector in the fragment clockwise from NotI to NotI. The structural gene may be inserted in the unique EcoRI site of this plasmid.

Detailed Description

The present invention provides improved expression cassettes for the expression of HSA, improved vectors and *Pichia pastoris* strains transformed with these improved cassettes and vectors.

Utilizing the present invention, HSA secretion levels of approximately 1-3.4 grams of authentic HSA per liter of fermentation broth have been obtained with an additional 15-88 µg HSA per mg of protein being present within the cell secretory pathway. This invention thus provides a means for the high level secretion of HSA. Achieving these levels of HSA production is a significant advancement over the prior production levels, since at the level of 1-3.4 grams per liter the recovery of HSA in high yields with high purities is possible.

To express the HSA structural gene, the gene must be operably linked to a 5' regulatory region and a 3' termination sequence, which forms an expression cassette which will be inserted into a host (usually a microorganism) via a vector (such as a circular plasmid or linear site-specific integrative vector). Operably linked as used in this context refers to a juxtaposition wherein the 5' regulatory region, structural gene, and 3' termination sequence are linked and configured so as to perform their normal function. 5' regulatory region or promoter as used herein means DNA sequences which respond to various stimuli and provide enhanced rates of mRNA transcription. 3' termination sequences are sequences 3' to the stop codon of a structural gene which function to stabilize the mRNA transcription product of the gene to which the sequence is operably linked (such as sequences which elicit polyadenylation). For the practice of this invention, it is preferred that the ATG of the structural gene be linked with as few intervening deoxyribonucleotides as possible to the 3' end of the 5' regulatory region, preferably about 11 or less deoxyribonucleotides and most preferably 8 or less deoxyribonucleotides. It is also preferred that the adenine and thymine content of the intervening deoxyribonucleotides be in the range of from about 55 percent to about 64 percent. Further, it appears that there are nucleotide preferences for certain specific

locations. Counting left from the ATG codon of the structural gene with the first position left being the -1 position it appears that adenine or cytosine is the most preferred deoxyribonucleotide, in the -2 position the most preferred deoxyribonucleotide is either adenine or thymine, in the -3 position the most preferred deoxyribonucleotide is adenine or thymine and the most preferred nucleotide at the -4 position is adenine, thymine or cytosine. Currently, it is preferred that the AOX1 or DAS1 5' regulatory regions having the restriction maps of Figures 3 and 4 or, the sequences provided as SEQ ID No: 1 and SEQ ID No: 2, respectively, be linked at their 3' end of the sequence to the ATG start codon of the HSA structural gene. Two examples of appropriate linkages for the AOX1 5' regulatory region are illustrated below.

Construct Designation	End of the 5' Regulatory Region for AOX 1	Deoxyribonucleotide intervening before ATG start condon
pHSA140	5' - TTCGAAACG	5' - AGGAATTC
pHSA413, pHSA313	5' - TTCGAAACG	5' - NONE

Several regulatory regions have been characterized and can be employed in conjunction with the expression of HSA in *Pichia pastoris*. Exemplary 5' regulatory regions are the primary alcohol oxidase (AOX1), dihydroxyacetone synthase (DAS1), and the p40 regulatory regions, derived from *Pichia pastoris* and the like. The presently preferred 5' regulatory regions employed in the practice of this invention are those characterized by their ability to respond to methanol-containing media, such regulatory regions selected from the group consisting of AOX1, and DAS1, disclosed by D. W. Stroman et al. in U.S. Patent 4,855,231, incorporated herein by reference. The most preferred 5' regulatory region for the practice of this invention is the AOX1 5' regulatory region.

3' termination sequences should be utilized in the expression cassette as discussed above. 3' termination sequences may function to terminate, polyadenylate and/or stabilize the messenger RNA coded for by the structural gene when operably linked to a gene, but the particular 3' termination sequence is not believed to be critical to the practice of the present invention. A few examples of illustrative sources for 3' termination sequences for the practice of this invention include but are not limited to the *Hansenula polymorpha* and *Pichia pastoris* 3' termination sequences. Preferred are those derived from *Pichia pastoris* such as those selected from the group consisting of the 3' termination sequences of AOX1 gene, DAS1 gene, p40 gene and HIS4 gene. Particularly preferred is the 3' termination sequence of the AOX1 gene.

Pichia pastoris may be transformed with a variety of HSA structural genes (in the inventive transformants discussed herein the HSA structural gene encodes both a signal sequence and a mature HSA protein). HSA structural genes have been sequenced by Lawn et al. Nuc. Acids Res. 9:6105 (1981), and Dugaiczky et al., Proc. Natl. Acad. Sci. USA 79:71 (1982). These genes may also be obtained by reisolation of the genes by the technique of Lawn et al., Dugaiczky et al. or synthesized *in vitro* by a custom gene manufacturer such as British Biotechnology, Ltd. One possible method of obtaining a HSA gene would be to screen a human liver cDNA library with oligonucleotide probes or screen a human liver cDNA expression library with anti-HSA antisera to identify HSA expressing human liver cDNAs. One suitable HSA structural gene is provided in SEQ ID NO: 3. Once a structural gene for HSA is recovered, it may be necessary to further tailor the gene. Following the isolation of a HSA structural gene, the gene is inserted into a suitable *Pichia pastoris* vector such as a plasmid or linear site-specific integrative vector.

Plasmid type vectors have long been one of the basic elements employed in recombinant DNA technology. Plasmids are circular extra-chromosomal double-stranded DNA found in microorganisms. Plasmids have been found to occur in single or multiple copies per cell. Included in plasmid DNA is the information required for plasmid reproduction, e.g. an autonomous replication sequence such as those disclosed by James M. Cregg in U.S. Patent 4,837,148, issued June 6, 1989, incorporated herein by reference. The autonomous replication sequences disclosed by Cregg provide a suitable means for maintaining plasmids in *Pichia pastoris*. Additionally one or more means of phenotypically selecting the plasmid in transformed cells may also be included in the information encoded in the plasmid.

Suitable integrative vectors for the practice of the present invention are the linear site-specific integrative vectors described by James M. Cregg, in U.S. Patent 4,882,279, issued November 21, 1989, which is incorporated herein by reference. These vectors comprise a serially arranged sequence of at least 1) a first insertable DNA fragment; 2) a selectable marker gene; and 3) a second insertable DNA fragment. An expression cassette containing a heterologous structural gene is inserted in this vector between the first and second insertable DNA fragments either before or after the marker gene. Alternatively, an expression cassette can be formed *in situ* if a regulatory region or promoter is contained within one of the insertable

fragments to which the structural gene may be operably linked.

The first and second insertable DNA fragments are each at least about 200 nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of the species to be transformed. The various components of the integrative vector are serially arranged forming a linear
 5 fragment of DNA such that the expression cassette and the selectable marker gene are positioned between the 3' end of the first insertable DNA fragment and the 5' end of the second insertable DNA fragment. The first and second insertable DNA fragments are oriented with respect to one another in the serially arranged linear fragment as they are oriented in the parent genome.

Nucleotide sequences useful as the first and second insertable DNA fragments are nucleotide sequences which are homologous with separate portions of the native genomic site at which genomic
 10 modification is to occur. For example, if genomic modification is to occur at the locus of the alcohol oxidase gene, the first and second insertable DNA fragments employed would be homologous to separate portions of the alcohol oxidase gene locus. Examples of nucleotide sequences which could be used as first and second insertable DNA fragments are deoxyribonucleotide sequences selected from the group consisting of
 15 the *Pichia pastoris* alcohol oxidase (AOX1) gene, dihydroxyacetone synthase (DAS1) gene, p40 gene and HIS4 gene. The AOX1 gene, DAS1 gene, p40 gene and HIS4 genes are disclosed in U.S. Patents 4,855,231 and 4,885,242 both incorporated herein by reference. The designation DAS1 is equivalent to the DAS designation originally used in U.S. Patents 4,855,231 and 4,885,242.

The first insertable DNA fragment may contain an operable regulatory region which may comprise the
 20 regulatory region utilized in the expression cassette. The use of the first insertable DNA fragment as the regulatory region for an expression cassette is a preferred embodiment of this invention. Figure 1 provides a diagram of a vector utilizing the first insertable DNA fragment as a regulatory region for a cassette. Optionally, as shown in Figure 1, an insertion site or sites and a 3' termination sequence may be placed immediately 3' to the first insertable DNA fragment. This conformation of the linear site-specific integrative
 25 vector has the additional advantage of providing a ready site for insertion of a structural gene without necessitating the separate addition of a compatible 3' termination sequence.

If the first insertable DNA fragment does not contain a regulatory region, a suitable regulatory region will need to be inserted linked to the structural gene, in order to provide an operable expression cassette. Similarly, if no 3' termination sequence is provided at the insertion site to complete the expression cassette,
 30 a 3' termination sequence can be operably linked to the 3' end of the structural gene.

It is also highly desirable to include at least one selectable marker gene in the DNA used to transform the host strain. This facilitates selection and isolation of those organisms which have incorporated the transforming DNA. The marker gene confers a phenotypic trait to the transformed organism which the host did not have, e.g., restoration of the ability to produce a specific amino acid where the untransformed host
 35 strain has a defect in the specific amino acid biosynthetic pathway, or provides resistance to antibiotics and the like. Exemplary selectable marker genes may be selected from the group consisting of the HIS4 gene (disclosed in U.S. Patent 4,885,242) and the ARG4 gene (disclosed in U.S. Patent 4,818,700 incorporated herein by reference) from *Pichia pastoris* and *Saccharomyces cerevisiae*, the invertase gene (SUC2) (disclosed in U.S. Patent 4,857,467 incorporated herein by reference) from *Saccharomyces cerevisiae*, or
 40 the G418^R/kanamycin resistance gene from the *E. coli* transposable elements Tn601 or Tn903.

Those skilled in the art recognize that additional DNA sequences can also be incorporated into the vectors employed in the practice of the present invention, such as, for example, bacterial plasmid DNA, bacteriophage DNA, and the like. Such sequences enable the amplification and maintenance of these
 45 vectors in bacterial hosts.

The insertion of the HSA structural gene into suitable vectors may be accomplished by any suitable technique which cleaves the chosen vector at an appropriate site or sites and results in at least one operable expression cassette containing the HSA structural gene being present in the vector. Ligation of the HSA structural gene may be accomplished by any appropriate ligation technique such as utilizing T4 DNA
 50 ligase.

The initial selection, propagation, and optional amplification of the ligation mixture of the HSA structural gene and a vector is preferably performed by transforming the mixture into a bacterial host such as *E. coli* - (although the ligation mixture could be transformed directly into a yeast host but, the transformation rate would be extremely low). Suitable transformation techniques for *E. coli* are well known in the art. Additionally, selection markers and bacterial origins of replication necessary for the maintenance of a vector
 55 in a bacterial host are also well known in the art. The isolation and/or purification of the desired plasmid containing the HSA structural gene in an expression system may be accomplished by any suitable means for the separation of plasmid DNA from the host DNA. Similarly the vectors formed by ligation may be tested, preferably after propagation, to verify the presence of the HSA gene and its operable linkage to a

regulatory region and a 3' termination sequence. This may be accomplished by a variety of techniques including but not limited to endonuclease digestion, gel electrophoresis, or Southern hybridization.

Transformation of plasmids or linear vectors into yeast hosts may be accomplished by suitable transformation techniques including but not limited to those taught by Cregg and Barringer, U.S. Patent 4,929,555; Hinnen et al., Proc. Natl. Acad. Sci. 75, (1978) 1929; Ito et al., J. Bacteriol. 153, (1983) 163; Cregg et al. Mol. Cell Biol. 5 (1985), pg. 3376; D. W. Stroman et al., U.S. Patent 4,879,231, issued November 7, 1989; or Sreekrishna et al., Gene, 59 (1987), pg. 115. Preferable for the practice of this invention is the transformation technique of Cregg et al. (1985). It is desirable for the practice of this invention to utilize linear vectors and select for insertions by Southern hybridization.

The yeast host for transformation may be any suitable methylotrophic yeast. Suitable methylotrophic yeasts include but are not limited to yeast capable of growth on methanol selected from the group consisting of the genera *Hansenula* and *Pichia*. A list of specific species which are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methylotrophs, 269 (1982). Presently preferred are methylotrophic yeasts of the genus *Pichia* such as the auxotrophic *Pichia pastoris* GS115 (NRRL Y-15851); *Pichia pastoris* GS190 (NRRL Y-18014) disclosed in U.S. Patent 4,818,700; and *Pichia pastoris* PPF1 (NRRL Y-18017) disclosed in U.S. Patent 4,812,405. Auxotrophic *Pichia pastoris* strains are also advantageous to the practice of this invention for their ease of selection. It is recognized that wild type *Pichia pastoris* strains (such as NRRL Y-11430 and NRRL Y-11431) may be employed with equal success if a suitable transforming marker gene is selected, such as the use of *SUC2* to transform *Pichia pastoris* to a strain capable of growth on sucrose or an antibiotic resistance marker is employed, such as resistance to G418.

Transformed *Pichia pastoris* cells can be selected for by using appropriate techniques including but not limited to culturing previously auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype ("methanol slow"), or culturing in the presence of an antibiotic which is toxic to the yeast in the absence of a resistance gene contained in the transformant.

Isolated transformed *Pichia pastoris* cells are cultured by appropriate fermentation techniques such as shake flask fermentation, high density fermentation or the technique disclosed by Cregg et al. in, High-Level Expression and Efficient Assembly of Hepatitis B Surface Antigen in the Methylotrophic Yeast, *Pichia Pastoris* 5 Bio/Technology 479 (1987). Isolates may be screened by assaying for HSA production to identify those isolates with the highest HSA production level.

Transformed strains, which are of the desired phenotype and genotype, are grown in fermentors. For the large-scale production of recombinant DNA-based products in methylotrophic yeast, a three stage, high cell-density, batch fermentation system is normally the preferred fermentation protocol employed. In the first, or growth stage, expression hosts are cultured in defined minimal medium with an excess of a non-inducing carbon source (e.g. glycerol). When grown on such carbon sources, heterologous gene expression is completely repressed, which allows the generation of cell mass in the absence of heterologous protein expression. It is presently preferred, during this growth stage, that the pH of the medium be maintained at about 5. Next, a short period of non-inducing carbon source limitation growth is allowed to further increase cell mass and derepress the methanol responsive promoter. The pH of the medium during this limitation growth period is adjusted to the pH value to be maintained during the production phase, which is generally carried out at about pH 5 to about pH 6, preferably either about pH 5.0 or about pH 5.8. Subsequent to the period of growth under limiting conditions, methanol alone (referred to herein as "limited methanol fed-batch mode") or a limiting amount of non-inducing carbon source plus methanol (referred to herein as "mixed-feed fed-batch mode") are added in the fermentor, inducing the expression of the heterologous gene driven by a methanol responsive promoter. This third stage is the so-called production stage.

The invention will now be described in greater detail in the following non-limiting examples.

Examples

General information pertinent to the Examples:

Strains

Pichia pastoris GS115 (his 4) NRRL Y-15851
E. coli JM103 delta (lac pro) thi rpsI (strA) supE endA sbcB hsdR.
E. coli K12 MC1061 NRR1-18016 (F⁻, araD139 delta (lac 1POZY)x74 galK galU hsr hsm(+)) rpsL delta (araABOIC leu)7697.

E. coli DG75' (hsd1, leu6, lacY, thr-1, supE44, tonA21, lambda⁻)

Buffers, Solutions and Media

5	The buffers, solutions, and media employed in the following examples have the compositions given below:	
	dH ₂ O	deionized H ₂ O that has been treated with a milli-Q (Millipore) reagent water system.
10	1M Tris buffer	121.1 g Tris base in 800 mL of H ₂ O; adjust pH to the desired value by adding concentrated (35%) aqueous HCl; allow solution to cool to room temperature before final pH adjustment, dilute to a final volume of 1 L.
	TE buffer	1.0 mM EDTA
15	SED	in 0.01 M (pH 8.0) Tris buffer
		1 M sorbitol
		25 mM EDTA
		50 mM DTT, added prior to use
20	SCE	--adjust to pH 8
		9.1 g sorbitol
		1.47 g Sodium citrate
		0.168 g EDTA
25	CaS	--pH to 5.8 with HCl in 50 ml dH ₂ O and autoclave
		1 M sorbitol
		10 mM CaCl ₂
		--filter sterilize
	SOS:	1 M sorbitol
30		0.3x YPD
	PEG	10 mM CaCl ₂
		20% polyethylene glycol-3350
		10 mM CaCl ₂
		10 mM Tris-HCl (pH 7.4)
		--filter sterilize
35	Solution A	0.2 M Tris-HCl (pH 7.5)
		0.1 M MgCl ₂
		0.5 M NaCl
		0.01 M dithiothreitol (DTT)
	Solution B	0.2 M Tris-HCl (pH 7.5)
40		0.1 M MgCl ₂
		0.1 M DTT
	Solution C (keep on ice)	4 μl solution B
		4 μl 10 mM dATP
		4 μl 10 mM dTTP
45		4 μl 10 mM dGTP
		4 μl 10 mM dCTP
		4 μl 10 mM ATP
		5 μl T ₄ ligase (2 U/μl)
		12 μl H ₂ O
50		Recipe for Solution C was modified from Zoller & Smith
	20X SSPE	4.4 g NaOH
		7.4 g Na ₂ EDTA
		27.6 g NaH ₂ PO ₄ · H ₂ O
55		210 g NaCl
		--pH adjusted to 7.5-8.0 with NaOH
		--H ₂ O to 1 liter
	50X Denhardt's	5 g Ficoll 400

		5 g Polyvinylpyrrolidone
		5 g BSA Fraction V
		H ₂ O to 500 ml
5	20X SSC	175.3 g NaCl
		88.2 g sodium citrate
		--pH to 7.0 with NaOH
		--H ₂ O to 1 liter
	LB Broth, 1 liter	5.0 g yeast extract
		10.0 g tryptone
10	10X Transfer Buffer	5.0 g NaCl
		96.8 g Trizma Base
		9.74 g glycine
		water to 1 liter
	Transfer Buffer for Tank	500 mls 10X Transfer Buffer
15		1000 mls methanol
		3500 mls water
	Western Buffer - for 1 liter	2.5 g gelatin put in solution by microwaving
		first in 100 mls water
		100 mls 10X PBS
20		1 ml 50% Tween-20
		4 mls 5% sodium azide
		dH ₂ O to 1 liter
	Coating Buffer	0.160 g Na ₂ CO ₃ (sodium carbonate)
		0.294 g NaHCO ₃ (sodium carbonate)
25		Add distilled water to 100 ml. Do not pH. (pH
		should be 9.5)
	Tris Buffered Saline (TBS)	26.1 g NaCl
		2.63 g Tris
		Add distilled water to 3 liters.
30		Adjust pH to 7.5 with HCl.
	Tris Buffered Saline/Tween (TBST)	1 liter of TBS
		2.5 ml of 20% Tween-20
	Blotto Buffer	50 g of non-fat dry milk (Carnation)
		1 g thimerosal (Sigma)
35		100 µl of antifoam (Sigma, 30% emulsion)
		2.5 ml of 20% Tween-20
		100 ml 10x PBS (house stock)
		Add distilled water to 1 liter
		Adjust pH to 7.5
40	Ligation Buffer	50 mM Tris-HCl (pH 7.4)
		10 mM MgCl ₂
		10 mM dithiothreitol
		1 mM ATP
	Phosphatase Buffer	50 mM Tris-HCl (pH 9.0)
45		1 mM MgCl ₂
		1 mM ZnCl ₂
		1 mM spermidine
	Bsu36I buffer	100 mM NaCl
		10 mM Tris-HCl (pH 7.4)
50		10 mM MgCl ₂
		100 µg/ml BSA
	Csp45I buffer	60 mM NaCl
		10 mM Tris-HCl, pH 7.5
		7 mM MgCl ₂
55		100 µg/ml BSA
	REact 1 buffer	50 mM Tris-HCl, pH 8.0
		10 mM MgCl ₂
		100 µg/ml BSA

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	REact 2 buffer	REact 1 buffer + 50 mM NaCl
	REact 3 buffer	REact 1 buffer + 100 mM NaCl
	HS buffer	50 mM Tris-HCl, pH 7.5
5		10 mM MgCl ₂
		100 mM NaCl
		1 mM DTT
		100 µg/ml BSA
	10X Basal Salts	42 mls Phosphoric Acid, 85%
10		1.8 g Calcium Sulfate • 2H ₂ O
		28.6 g Potassium Sulfate
		23.4 g Magnesium Sulfate • 7H ₂ O
		6.5 g Potassium Hydroxide
	Ptm ₁ Trace Salts Solution	6.0 g Cupric Sulfate • 5H ₂ O
15		0.08 g Sodium Iodide
		3.0 g Manganese Sulfate • H ₂ O
		0.2 g Sodium Molybdate • H ₂ O
		0.02 g Boric Acid
		0.5 g Cobalt Chloride
20		20.0 g Zinc Chloride
		65.0 g Ferrous Sulfate • H ₂ O
		0.20 g Biotin
		5.0 mls Sulfuric Acid
	YPD (yeast extract peptone dextrose medium)	10 g bacto yeast extract
25		20 g peptone
		10 g dextrose
		water to 1 liter
	MGY (minimal glycerol medium)	13.4 g yeast nitrogen base with ammonium sulfate, and without amino acids
30		400 µg biotin
		10 ml glycerol
		water to 1 liter
35	MM (minimal methanol medium)	Same as MGY, except that 5 ml methanol is used in the place of 10 ml glycerol.
	SDR (supplemented dextrose regeneration medium):	13.4 g yeast nitrogen base with ammonium sulfate and without amino acids
40		400 µg biotin
		182 g sorbitol
		10 g glucose
		2 g Histidine assay mix (Gibco)
45		50 mg glutamine
		50 mg methionine
		50 mg lysine
		50 mg leucine
		50 mg isoleucine
50		10 g agarose
		water to 1 liter
	BMGR (Buffered minimal glycerol-enriched medium)	100 ml/liter Potassium phosphate buffer, (pH 6.0)
55		13.4 grams/liter Yeast nitrogen base with ammonium sulfate
		400 µg/liter biotin
		10 ml/liter glycerol

5	Amino acids	glutamic acid, methionine, lysine, leucine and isoleucine: each at 5 mg/liter; all the other amino acids except histidine at 1 mg/liter
	Nucleotides	adenine sulfate, guanine hydrochloride, uracil, and xanthine, each at 40 µg/liter
10	Vitamins	thiamine hydrochloride, riboflavin, and calcium pantothenate, each at 2 µg/liter; pyridoxine hydrochloride and nicotinic acid, each at 4 µg/liter; pyridoxamine hydrochloride and pyridoxal hydrochloride, each at 1 µg/liter; para-amino benzoic acid at 0.3 µg/liter; folic acid at 0.03 µg/liter
15	Trace minerals	magnesium sulfate at 800 µg/liter; ferrous sulfate at 40 µg/liter; manganese sulfate at 80 µg/liter; sodium chloride at 40 µg/liter
20	BMGY (Buffered minimal glycerol-complex medium)	100 ml/liter potassium phosphate buffer, (pH 6.0) 13.4 grams/liter yeast nitrogen base with ammonium sulfate and without amino acids biotin at 400 µg/liter glycerol at 10 ml/liter yeast extract at 10 g/liter peptone at 20 g/liter
25	BMMR (Buffered minimal methanol-enriched medium)	Same as BMGR, with the exception that 5 ml methanol/liter is added in the place of glycerol
30	BMMY (Buffered minimal methanol -complex medium)	Same as BMGY, with the exception that 5 ml methanol/liter is added in the place of glycerol
35		

Techniques

Suitable techniques for recombinant DNA lab work may be found in many different references including but not limited to: Methods in Enzymology, (Orlando, FL: Academic Press, Inc.), particularly Volume 152, published as, Guide to Molecular Cloning Techniques, by Berger and Kimmel (Orlando, FL: Academic Press, Inc., 1987) and Molecular Cloning/A Laboratory Manual, by Sambrook et al., 2d ed. (Cold Spring Harbor Laboratory Press, 1989) and which are all hereby incorporated by reference.

Example I

Construction of mHSA13

Mutagenesis of HSA Structural Gene Insert

DNA encoding HSA was obtained from pHSA13, disclosed in European Patent Application 0 344 459, herein incorporated by reference, by EcoRI digestion. A 2069 bp fragment was recovered by electrophoresis on a 1% agarose gel. The DNA was mutagenized by the following procedure to make the following changes: 1) an EcoRI restriction site was added immediately prior to the ATG of the HSA signal sequence, and 2) an EcoRI restriction site was added immediately adjacent to the TAA stop codon in the HSA cDNA.

The oligonucleotides employed in the mutagenesis were:

- 1) 5' mutagenesis to add EcoRI site, mutagenizing nucleotide sequence:

5' CCC TCA CAC GCC TTT GAA TTC ATG AAG TGG GTA ACC 3' (SEQ ID NO:4)

2) 3' mutagenesis to add EcoRI site, mutagenizing nucleotide sequence:

5' GCC TTA GGC TTA TAA GAA TTC AGT TTA AAA GCA TCT CAG 3' (SEQ ID NO:5)

and were synthesized using an Applied Biosystems DNA Synthesizer, Model 380A using cyanoethyl-phosphoramidite chemistry.

1.2 µg of double-stranded m13mp10 were digested with EcoRI and dephosphorylated and ligated with 450 ng of the previously isolated 2069 bp fragment containing the HSA structural gene.

The ligation mixture was transformed into competent JM103 cells (competent JM103 were prepared as described in Example II for MC1061 cells). The mixture was then plated on LB media containing IPTG and X-gal and the plates screened for clear plaques. DNA was recovered from transformants and digested with Hind III. The correct phage demonstrated bands of 7369 and 1950 bp and was called mHSA13.

A. A large scale miniprep was performed on positive plaques which had been incubated for approximately 7 hours in 2 mls of L media. 25 mls of LB media was inoculated with 250 µl of freshly grown JM103 cells. The culture was grown for 1 hour and inoculated with 100 µl of the 7 hour old plaque culture. The culture was then grown overnight. The culture was centrifuged twice at 10,000 rpms for 10 minutes on a Sorvall RC-5B rotor SS34 to clear the supernatant. 3.5 ml of 20% PEG/2.5 M NaCl was added to the culture and it was incubated for 5 hours at 4°C. The culture was then centrifuged again as above for 10 minutes. The supernatant was discarded and the pellet was resuspended in 2 mls of TE buffer. The pellet was then extracted with phenol, equilibrated with TE, extracted with phenol/chloroform, extracted twice with CHCl₃ and once with ether. 8 M LiCl was added to attain a final concentration of 0.8 M. 3 volumes of ethanol were added and the solution left overnight at -20°C to precipitate the DNA present. The solution was next centrifuged for 10,000 rpms for 10 minutes as previously described and rinsed with 70% ethanol. The precipitate was resuspended in 150 µl of 10 mM Tris (pH 7.4).

B. One pmole of M13 recombinant template was mixed with 20 pmole of oligonucleotide 1 (for 5' mutagenesis to create an EcoRI site), 1 µl of solution A and dH₂O was added to give a final volume of 10 µl. The sample was incubated at 65°C for 5 minutes, and the temperature was then reduced to 37°C for 30 minutes.

C. The following was then added to the sample:

30

Solution B	1 µl
10 mM dATP	1 µl
10 mM dCTP	1 µl
10 mM dGTP	1 µl
10 mM dTTP	1 µl
5 u/µl Klenow	2 µl
dH ₂ O	3 µl
	<u>20 µl</u>

35

40 and allowed to incubate at 15°C for at least 4-6 hours.

D. The sample was then diluted 1:40 with dH₂O. 5 µl was used to transform 6 tubes of competent JM103 cells (200 µl each). The transformed JM103 cells were plated on rich media in a soft agar overlay.

E. The positive plaques were then screened for by filter hybridization.

45 A hybridization probe of 15 pmole of complementary oligonucleotide in a total volume of 25 µl total volume was heated to 65°C for 10 minutes. 3 µl 10X kinase buffer (Maniatis), 1 µl γ-ATP and 1 µl polynucleotide kinase (100 u/µl) were added to the sample. The sample was incubated for 1 hour at 37°C and run through G-50 fine Sephadex. The first peak off the column was collected.

50 Nitrocellulose filters were prepared for hybridization with the above probe by placing and orienting the filters on the transformation plates for 5-10 minutes. The filters were then removed from the plates and floated on a denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 3 minutes with the backside on top of the solution. The filters were then submerged in the denaturing solution for 5 minutes. The nitrocellulose filters were transferred to a neutralizing solution (1 M Tris•HCl, pH 8; 1.5 M NaCl) for 5 minutes. The neutralized filter was then transferred to 2XSSC (1XSSC is 150 mM NaCl, 15 mM NaCitrate) for 5 minutes. The filter was then air dried and baked for 1 hour at 80°C under a vacuum. The filters were prehybridized for 1 hour at 65°C in a sealed plastic bag containing 5 ml of hybridization buffer filter, 10X Denhardtts (1X Denhardtts is 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin) 0.5% SDS and 5XSSPE. The hybridization buffer was replaced with 5 ml/filter of fresh hybridization

55

buffer. The screening oligonucleotides utilized were as follows:

- 1) 5' mutagenesis the screening oligonucleotide was
5' GCC TGG GAA TTC ATG AAG 3' (SEQ ID NO:6)
- 2) 3' mutagenesis the screening oligonucleotide was
5' TTA TAA GAA TTC AGT TTA 3' (SEQ ID NO:7)

The previously prepared screening oligonucleotide was first incubated at 65°C for 5 minutes, and then enough probe was added to the fresh hybridization buffer containing the filter to give 1X10⁶ cpm/ml. Hybridization was performed at 5°C below the calculated melting temperature of the probe for 4 hours.

The filters were then washed three times for 10 minutes each with 6XSSC at room temperature. The filters were finally washed one time with 6XSSC at the hybridization temperature. The filters were placed on a 3 MM Whatman paper to dry, and then exposed to film (marked for orientation) overnight.

Three positive plaques were each picked and grown separately in 2 mls of LB broth at 37°C for 5 hours.

F. Mini template preps were performed on each of these positive plaques.

One ml of the plaque culture was transferred into an Eppendorf tube and centrifuged for 5 minutes in a Eppendorf Model 5414 Centrifuge. 800 µl of the supernatant was recovered and 200 µl of 20% PEG with 2.5M NaCl was added thereto. The supernatant was incubated at room temperature for 10 minutes. The supernatant was centrifuged for 10 minutes in the Eppendorf centrifuge previously used. The supernatant was removed by aspiration and the pellet formed by centrifuging was redissolved in 200 µl TE (10 mM Tris, pH 7.4; 1 mM EDTA). The redissolved pellet was then phenol/chloroform extracted and the template DNA in the upper aqueous phase was precipitated by the addition of a LiCl solution until a 0.8 M concentration was reached. To the solution was added 2 1/2-3 volumes of ethanol and precipitated on dry ice for 5 minutes. The precipitate was centrifuged for 10 minutes in the previously mentioned Eppendorf centrifuge. The final volume was brought up to 150 µl with TE.

G. 200 µl of competent JM103 cells were transformed with the recovered DNA. 1 µl and 1 µl of a 1/10 dilution of the isolated phase DNA was used in the transformation.

H. The transformation mixture was plated and plaques were screened with oligonucleotides as previously described in step E.

I. A large scale miniprep was performed on positive plaques which had been incubated for approximately 7 hours in 2 mls of L media. 25 mls of LB media was inoculated with 250 µl of freshly grown JM103 cells. The culture was grown for 1 hour and inoculated with 100 µl of the 7 hour old plaque culture. The culture was then grown overnight. The culture was then centrifuged twice at 10,000 rpms for 10 minutes on a Sorvall RC-5B rotor SS34 to clear the supernatant. 3.5 ml of 20% PEG/2.5M NaCl was added to the culture and it was incubated for 5 hours at 4°C. The culture was then centrifuged again as above for 10 minutes. The supernatant was discarded and the pellet was resuspended in 2 mls of TE buffer. The pellet was then extracted with phenol, equilibrated with TE, extracted with phenol/chloroform extracted twice with CHCl₃ and once with ether. 8 M LiCl was added to attain a final concentration of 0.8 M LiCl. 3 volumes of ethanol were added and the solution left overnight to precipitate the DNA present. The solution was next centrifuged for 10,000 rpms for 10 minutes as previously described and rinsed with 70% ethanol. The precipitate was resuspended in 150 µl of 10mM Tris (pH 7.4).

J. The positive plaques were then sequenced by dideoxy sequencing to find the M13 constructs with the correct mutations.

K. Repeat steps B-J using M13 constructs with the correct 5' mutations as templates and the second oligonucleotide as a primer for 3' mutagenesis. The correct mutation was designated mHSA140.

L. Recover RF DNA of mHSA140 using the alkaline lysis method of Maniatis.

Example II

Construction of the pHSA140 Expression Vectors

pA0804 is available in an *E. coli* host from the Northern Regional Research Center of the United States Department of Agriculture, Peoria, Illinois, accession number B-18114. pA0804 is recovered by isolating the plasmid DNA, digesting with *EcoRI*, gel electrophoresing to recover the ~7.5 kb fragment, which is linear pA0804 cut at its unique *EcoRI* site.

pA0804 is a vector capable of site-specific disruption of the *Pichia pastoris* AOX1 locus. It contains the following elements: the AOX1 promoter and transcription terminator separated by a unique *EcoRI* cloning site; the wild-type *Pichia* HIS4 gene; a genomic segment of DNA from the 3' end of the AOX1 locus downstream of the transcription terminator; and sequences necessary for selection and replication in a

bacterial host. The components are arranged such that a BglII restriction digest of the plasmid releases a DNA fragment containing the expression cassette and selective marker whose ends are homologous to a continuous portion of the genome, the AOX1 locus, and can be stably inserted into the chromosome during transformation. Additionally the ampicillin resistance gene and the *ori* from plasmid pBR322 are also

5 contained in the pA0804 plasmid.

A vector containing the gene coding for the production of HSA was constructed from pA0804 and mHSA140. pA0804 was digested with EcoRI and the ends were dephosphorylated by treatment with alkaline phosphatase (1 U enzyme at 37°C for 1 hr. in 50 mM Tris•Cl, pH 9.0, 1 mM MgCl₂, 100 mM ZnCl₂, 1 mM spermidine). mHSA140 was also digested with EcoRI, and a 1829 bp fragment encoding HSA was released.

10 This fragment was purified using 0.8% preparative agarose gel electrophoresis. 60 ng of the fragment were ligated to 240 ng of pA0804 by incubation at 23°C for 1 hr in 66 mM Tris•Cl, pH 7.4, 5 mM MgCl₂, 5 mM dithiothreitol, 1 mM ATP, with 1 Weiss Unit of T4 ligase in a 10 µl reaction volume. The ligation reaction was used to transform competent MC1061 cells to ampicillin resistance.

MC1061 was rendered competent for transformation in the following manner. A mid-log culture (50 ml) of *E. coli* MC1061 was harvested by centrifugation in an IEC DPR 600 clinical centrifuge at 3,000 rpm for 5 min at 4°C and washed in 10 mM NaCl. The culture was resuspended in 25 ml of 50 mM CaCl₂ for 30 min at 0°C. The cells were centrifuged as above and resuspended in 2 ml of 50 mM CaCl₂.

For transformation, the ligation reaction was added to 200 µl of the competent cell suspension and incubated at 0°C on ice for 15 minutes, heat shocked at 37°C for 5 minutes and incubated at 23°C for 5 minutes. The cells were plated directly onto LB agar plates containing 50 µg/ml ampicillin. The plates were incubated at 37°C for 10-16 hours. The resulting colonies were Amp^R. The resistant colonies were harvested and characterized by restriction digestion. Cells were grown in 5 ml of L-broth containing 50 µg/ml ampicillin for 5 hr at 37°C and DNA was prepared by the method of Birnboim and Doly [Nucleic Acids Research 7:1513 (1979)]. The minipreps displaying 4750, 3000 and 1900 bp fragments upon PvuII

25 digestion were chosen and designated pHSA140.

Example III

Transformation of *Pichia pastoris*

30 *Pichia pastoris* strains containing the vectors described in Example II were generated in the following manner. Methanol utilization deficient (Mut⁻ or methanol slow) and wild type methanol utilization (Mut⁺ or metaanol normal) strains were developed.

Pichia pastoris strain GS115 (his4; NRRL Y-15851) was transformed using the spheroplast transformation technique described by Cregg et al., Bio/Technology 5:479-485 (1987). See also U.S. 4,879,231.

35

A. Mut⁺ Strains

To direct integration of the vector to the AOX1 locus, 2 and 10 µg of SacI- digested pHSA140 were separately transformed into 5 of OD₆₀₀ (or 25 x 10⁷ cells) of GS115. Transformants were regenerated on minimal media and screened for the His⁺ phenotype. Several His⁺ transformants were then screened by Southern analysis for the site of integration and vector copy number (Example V).

40

B. Mut⁻ Strains

45 To develop Mut⁻ strains, in which the HSA expression cassette integrates into and disrupts the AOX1 structural gene, vector pHSA140 was digested with PvuI and then partially digested with BglII. The digest was then size fractionated on a 0.8% agarose gel and DNA in the size range of 6.0-9.0 kb was isolated (the expression cassette was expected to be ~ 7.4 kb). 5µg of this DNA were used to transform 5 OD₆₀₀ (25 x 10⁷ cells) of GS115 by the spheroplast method. His⁺ cells were identified and then screened for the Mut⁻ phenotype as follows.

Transformants were pooled by scraping the surface of the plate in the presence of sterile distilled water and sonicated at low output for 15 seconds. They were subsequently diluted to an A₆₀₀ = 0.1 and plated at dilutions of 10⁻³ and 10⁻⁴, in duplicate onto minimal plates containing glycerol as the carbon source, and incubated at 30°C for 2-3 days. They were then replica-plated onto minimal plates to which 100 µl of methanol was added in the vapor phase. After a 24-hour incubation at 30°C, it was apparent that 4% of the transformants were growing more slowly on methanol than the rest of the transformants. Five of the His⁺ Mut⁻ isolates were examined by Southern analysis (Example V).

55

Example IVYeast DNA Miniprep

5 10^4 cells/ml were seeded in 5 ml YPD at 30°C overnight and then pelleted using a Damon IEC DPR600 clinical centrifuge at 3,000 rpm for 5 minutes. The pellet was resuspended in 0.5 ml of 1 M sorbitol, 0.1 ml 0.5 M EDTA, pH 8 and the sample transferred to a 1.5 ml microfuge tube. 0.02 ml of 2.5 mg/ml Zymolyase 100,000 (Miles Laboratories) was added, and the sample was incubated at 37°C for 60 minutes. The cells were pelleted using the microfuge for 1 minute at high speed, and resuspended in 0.5 ml of 50 mM Tris•Cl, pH 7.4 and 20 mM EDTA. 0.05 ml of 10% SDS was added, the sample mixed, and incubated at 65°C for 30 minutes. 0.2 ml of 5 M potassium acetate, pH 5.2, was added and the sample was incubated on ice for 60 minutes. The sample was again spun in a microfuge at high speed for 5 minutes.

15 The supernatant was transferred to a fresh 1.5 ml microfuge tube and 1 volume of isopropanol at room temperature was added. The sample was mixed and allowed to sit at room temperature for 5 minutes, then spun very briefly (10 seconds) in a microfuge at high speed. The supernatant was poured off and the pellet air dried. After resuspending the pellet in 0.3 ml of 10 mM Tris•Cl, pH 7.4 and 1 mM EDTA, 15 µl of a 1 mg/ml solution of pancreatic RNase was added, and the sample was incubated at 37°C for 30 minutes. 0.03 ml of 3 M sodium acetate was added, the sample mixed, and 0.2 ml of isopropanol added. The sample was spun in a microfuge at high speed to pellet the DNA. The supernatant was then poured off, the pellet dried and resuspended in 0.1-0.3 ml of 10 mM Tris•Cl, pH 7.4 and 1 mM EDTA. (Note: Before using the DNA in a restriction digest, it may be necessary to spin the solution for 15 minutes at high speed in the microfuge to remove any insoluble material which may inhibit the digestion).

Example V

25

Strain Characterization

DNA was prepared from the transformed *Pichia* cells (Example III) and from untransformed host *Pichia* cells as described in Example IV, and digested with EcoRI. The samples were electrophoresed on 0.8% agarose gels, and Southern blots were performed (Maniatis et al, 1982). The filters were hybridized with an AOX1 specific probe or with a HIS4 specific probe to determine where integration had occurred. The site of integration was determined by comparing the spectrum of hybridization of a given transformant with the wild type strain. Any alteration in the size of the wild type band was evidence of integration at that locus. A summary of the Southern hybridizations and strain characterization for the strains chosen for further analysis is below.

Table I

Strain Name	Site of Integration	Vector Copy Number
G + HSA140S1	AOX1	one
G + HSA140S4	AOX1	two
G + HSA140S3	AOX1	>two
G-HSA140S1	AOX1	one

Fermentor Growth of HSA-Expressing *Pichia* Strains

50 Inocula were prepared from selective plates and grown overnight at 30°C in buffered YNB containing 2% glycerol to an OD₅₀₀ of 0.5-10.0. An aliquot of 5-50 ml of the overnight culture was added to a 2-liter capacity fermentor, and the repressed growth phase continued in 5X basal salts containing 5 ml/L of PTM₁ salts at 30°C. The pH was maintained at 5.0 by the addition of 40% (v/v) ammonium hydroxide, and foaming was controlled by the addition of 5% (v/v) Struktol antifoam. Dissolved oxygen was maintained above 20% by increased aeration and agitation as needed. The temperature was maintained at about 30°C. This batch growth phase continued for 20-30 hours until the glycerol was exhausted. The fermentation was then continued in either a methanol-limited fed-batch mode for Mut⁺ strains or a methanol-excess fed-batch mode for Mut⁻ strains.

a. Mut⁺ fermentation; methanol-limited fed batch

Run 544: G + HSA140S1 (1 copy)

Run 557: G + HSA140S4 (2 copy)

5 Run 545: G + HSA140S3 (>2 copy)

In order to continue building cell density as well as to prevent the accumulation of excess ethanol in the fermentor, the AOX1 promoter was derepressed to allow the expression of a small amount of alcohol oxidase before induction by the addition of methanol. This derepression was achieved by growth under glycerol-limited conditions at about pH 5.0. Following exhaustion of the glycerol in the initial growth phase, a
10 50% (w/v) glycerol feed (containing 12 ml/L of PTM₁ trace salts) was initiated at a rate of 8-16 ml/hour and continued until approximately 120-140 ml had been added. Full expression of the AOX1 promoter was then induced by the initiation of a methanol feed (100% MeOH plus 12 ml/L PTM₁ trace salts) at 1 ml/hour. The methanol feed was maintained for several hours until the culture responded to methanol limitation. This response was expressed as a sudden rise in dissolved oxygen upon a brief cessation of the methanol feed.
15 The methanol feed was then increased over an 8-12 hour period until a rate of 5.5 ml/hour was achieved. Fermentation was continued under these conditions for 82, 96 or 98 hours on methanol before the culture was harvested.

b. Mut⁻ fermentation; methanol excess fed batch

20

The Mut⁻ fermentations were conducted as described for the Mut⁺ fermentations, except the MeOH feed was increased after 4 hours of 1 ml/hr feed to 3-4 ml/hr, to give a residual methanol concentration less than 0.5%.

25 Quantification of HSA Secreted into Growth Media

a. ELISA

The ELISA procedure for human serum albumin requires the following reagents: Human Albumin (obtained from Cappell, Organon Teknika), Goat anti-HSA antibody (obtained from Atlantic Antibodies), Goat anti-HSA antibody, peroxidase conjugated (obtained from Cappell, Organon Teknika), and O-phenylenediamine (OPD), dichloride salt (obtained from Sigma, 10 mg/tablet). The HSA was reconstituted following the manufacturers directions. In this case (lot #26706) 3.0 ml of distilled water was added to the contents of the vial (the final concentration was 18.8 mg/ml). 29 aliquots of 100 μ l each were labeled and quickfrozen.
35 16 aliquots were formed by diluting 100 μ l of 18.8 mg/ml with 1.780 ml PBS (final 1.0 mg/ml). The 16 aliquots of 100 μ l were labeled and quickfrozen.
100 more aliquots were formed by diluting 100 μ l of 1 mg/ml with 9.9 ml PBS (final 10 μ l). The 100 aliquots of 100 μ l each, were then labeled and quickfrozen. This dilution was used to begin the standard curve of dilutions. The goat anti-HSA was supplied in solution. The goat anti-HSA reagent was divided into 50 μ l
40 aliquots, labeled and quickfrozen. Goat anti-HSA conjugated to peroxidase was reconstituted by the addition of 2.0 mls of distilled water (final concentration 23 mg/ml). 50 μ l of aliquots of the goat anti-HSA conjugate were then labeled and quickfrozen.

ELISA PROCEDURE:

45

Note: Use buffers at room temperatures only.

1. Make up coating buffer immediately before use. Dilute goat anti-HSA antibody 1:500. Add 200 μ l of this solution to each well. Parafilm tightly and incubate one hour at 37 °C.
2. Sharply flick contents of plate into sink. Wash 3 times with TBST. Wash 2 times with distilled water.
- 50 3. Add 200 μ l blotto buffer to all wells. Parafilm tightly. Incubate overnight at 37 °C.
4. Next morning, flick contents of wells into sink. Wash 3 times with TBST. Wash 2 times with distilled water.
5. Add 100 μ l of TBST to all wells.
6. Dilute stock 10 μ g/ml HSA standard with TBST.
- 55 S = stock from freezer = 10 μ g/ml
- SS = substock = 1:100 of S = 10,000 pg/100 μ l
- S⁻¹ = 1:10 of SS = 1,000 pg/100 μ l
- Dilute SS, 1:1 = 5,000 pg/100 μ l

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Dilute SS, 1:3.3 = 3,000 pg/100 µl

Dilute SS, 1:5 = 2,000 pg/100 µl

Dilute S⁻¹, 1:1 = 500 pg/100 µl

Dilute S⁻¹, 1:5 = 200 pg/100 µl

5 Dilute 3,000 pg/100 µl 1:1 = 1,500 pg/100 µl

8. Add 100 µl sample dilutions and standard curve dilutions to each well.

9. Parafilm tightly and incubate 2 hours at 37 ° C.

10. Wash five times with TBST. Wash two times with distilled water.

10 11. Dilute goat anti-HSA conjugate 1:2000 with blotto buffer. Incubate for two hours at room temperature in the dark.

12. Wash three times with TBST. Wash two times with distilled water.

13. Immediately before use: add one pellet of OPD to 3 mls of distilled water in a dark container. Pipet 21 mls of water into a 50 ml Falcon tube. Add 3 mls OPD solution to the Falcon tube, add 10 µl of 30% H₂O₂, and mix.

15 Add 200 µl of this solution to each well. Parafilm tightly and incubate 10 minutes in the dark. Stop the reaction by addition of 50 µl 4.5 M sulfuric acid.

14. Read on ELISA reader at 492 nm using filter 4.

b. Data

20

The level of HSA secreted from each of the strains, and other information pertinent to the fermentations, is provided in Table II:

Table II

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Fermentation of HSA Strains						
Run	Strain	Copy Number	Integration Site	Hours on MeOH	Cell Density (Wet) g/l	HSA in Broth g/l
537	G-HSA140S1	1	AOX1/Mut ⁺	98	445	0.971
544	G + HSA140S1	1	AOX1/Mut ⁺	96	415	0.964
557	G + HSA140S4	2	AOX1/Mut ⁺	82	450	0.754
545	G + HSA140S3	>2	AOX1/Mut ⁺	96	353	0.185

35

Characterization of Recombinant Product

a. Gel analysis

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Samples of fermentation broths of fermentation runs 537, 544 and 545 were withdrawn at different time points during the induction phase and analyzed by SDS gels and Coomassie blue staining. 5 µl of a 10-fold dilution of fermentor broth (equivalent to 0.5 µl) were applied to the gel. The relative intensity of the stained bands of rHSA (recombinant HSA) and the HSA standard confirmed the high (gram/liter) rHSA levels found by ELISA. In addition, the rHSA from all three fermentations and at all time points analyzed showed identical mobility with the HSA standard (69 Rd). The fact that the rHSA was the major protein species secreted by all HSA expression strains suggests high initial purity (>90%) of the rHSA secreted into the growth medium. Another protein species which migrates at approximately 45 Kd could also be detected in increasing intensity with respect to fermentation time. This protein species may be produced in a secondary processing event during secretion or as a proteolytic product post-secretion by proteases secreted into the fermentation broth, or both. It is related to rHSA, as it was detectable on Western blots by HSA specific polyclonal antisera.

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b. N-terminal sequence

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Protein sequence of the N-terminal region of secreted rHSA was obtained on a dialysed sample of fermentor broth. The sequence was determined on an Applied Biosystems Model 470A protein sequencer. The phenylthiohydantoin (PTH) derivatives of the amino acids were identified by high performance liquid

chromatography with an Applied Biosystems Model 120A analyzer. The results showed that the rHSA N-terminus is aspartic acid, consistent with the N-terminal amino acid of HSA. There does not appear to be any other precursor type of rHSA. The balance of the sequence determined was identical to the known sequence for HSA.

Example VI

Construction of HSA expression vector pHSA313

The pHSA313 vector was constructed to provide a vector with an exact linkage between the 3' end of the native AOX1 5'regulatory region (promoter) and the start codon of the HSA structural gene.

A. Creation of pHSA113bC1a

About 200 ng of pHSA113 (disclosed in European Patent Application 0 344 459 and shown in Figure 7) was digested at 37°C for 1 hour with 1 unit of Clal in 20 µl of REact 1 buffer. The digestion mixture was brought to 100 µl with water and extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 V/V), followed by extracting the aqueous layer with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA in the aqueous phase was precipitated by adjusting the NaCl concentration to 0.2 M and adding 3 volumes of cold ethanol. The mixture was allowed to stand on ice (4°C) for 10 minutes and the DNA precipitate was collected by centrifugation for 30 minutes at 10,000 x g in a microfuge at 4°C. The DNA pellet was washed 2 times with 70% aqueous cold ethanol. The washed pellet was vacuum dried and dissolved in 10 µl water to which 2 µl of 10 x ligation buffer, 2 µl of 1 mg/ml BSA, 6 µl of water and 1 unit T₄ DNA ligase were added. The mixture was incubated overnight at 4°C and a 10 µl aliquot was used to transform *E. coli* DG75' (Maniatis, et al.) to obtain pHSA113ΔCla, which represents the deletion of HIS4 and 3'AOX1, along with small stretches of pBR322 sequences used to link these sequences. The deletion of the HIS4, 3'AOX1 and pBR322 sequences removes one of two Csp45I sites present in the pHSA113 vector. The remaining Csp45I site is in the AOX1 5'regulatory region (promoter).

B. Creation of pXHSA113ΔCla

Digest 5 µg of pHSA113ΔCla for 1 hour at 37°C with 10 units of BstEII in 100 µl of REact 2 buffer. The digestion mixture was extracted with phenol and precipitated as detailed in step A. The DNA precipitate was dissolved in 100 µl of Csp45I buffer and digested at 37°C for 2 hours in the presence of 10 units of Csp45I. The digested DNA was then phenol extracted and precipitated as described in step A. The DNA precipitate was dissolved in 20 µl of water and 10 µl aliquots were loaded on 2 neighboring wells of a 0.9% agarose gel. Following electrophoresis, the gel portion corresponding to one of the lanes was stained and this was used to locate the position of the Csp45I-BstEII fragment of pHSA113ΔCla in the unstained lane. The gel portion containing the larger Csp45I-BstEII fragment of pHSA113ΔCla was excised from the gel. The gel portion containing the larger Csp45I-BstEII fragment was electroeluted into 500 µl of 5 mM EDTA, pH 8.0. The DNA solution was phenol extracted as detailed in step A and the DNA precipitate was dissolved in 100 µl water. The larger Csp45I-BstEII fragment was then ligated with the BstEII-Csp45I oligonucleotide linker described below. An aliquot (10 µl) of the Csp45I-BstEII fragments was ligated overnight at 4°C with 20 ng of annealed linker oligonucleotides 5'-CGAAACG ATG AAG TGG (SEQ ID NO:8) and 5'-GTTACCCACT-TCATCGTTT (SEQ ID NO:9) in 20 µl ligase buffer containing 100 µg/ml BSA and 1 unit of T₄ DNA ligase. The ligation mixture was used to transform *E. coli* DG75' to obtain pXHSA113ΔCla. The pXHSA113ΔCla vector by virtue of the linker described above has an exact linkage between the 3' end of the native AOX1 5' regulatory region (promoter) and the HSA ATG start codon with no extraneous DNA sequences.

C. Creation of pHSA313

1 µg of pXHSA113ΔCla was digested for 4 hours at 37°C with Clal in 100 µl of REact 1 buffer. Following digestion the reaction mixture was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in a 200 µl reaction volume for 30 minutes at 37°C. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated and dissolved in water at a concentration of approximately 10 ng/µl as described in step A and stored at -20°C.

1 µg of pA0807N (Figure 8, construction of which is described in European Patent Application 0 344 459) was digested for 4 hours at 37°C with PstI in 100 µl of REact 2 buffer. The digested DNA was

adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in a 200 μ l reaction volume for 15 minutes at 55 °C. At the end of 15 minutes another 10 units of phosphatase was added and incubated for 15 minutes. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated as described in step A. DNA was digested for 4 hours at 37 °C with 5 units of Clal in 100 μ l REact 1 buffer containing 100 μ g/ml BSA, followed by phenol extraction and precipitation of DNA as outlined in step A. The DNA precipitate was dissolved in water at a concentration of approximately 20 ng/ μ l. This Clal fragment contains the HIS4 gene and 3' AOX1 second insertable sequence.

Approximately 100 ng (10 μ l) of Clal cleaved-phosphatased pXHSA113 Δ Clal was mixed with approximately 80 ng of PstI digested-phosphatased and Clal-cleaved pA0807N (4 μ l), 4 μ l of 5X ligase buffer, 2 μ l of 1 mg/ml BSA and ligated overnight at 4 °C using 1 unit of T₄ DNA ligase. The ligation mixture was used to transform E. coli DG75' to obtain pHSA313. The pHSA313 plasmid from this ligation contains the complete pXHSA113 Δ Clal sequence linked to the HIS4 gene and the AOX1 3' second insertable sequence derived from pA0807N. The relative orientation of the components of the pHSA313 plasmid is the same as that shown in Figure 7 for plasmid pHSA113.

Example VII

Construction of 5' & 3' exact HSA expression plasmid pHSA413

The pHSA413 vector was constructed to provide a vector with an exact linkage between the 3' end of the AOX1 5' regulatory region and the start codon of the HSA structural gene as well as an exact linkage between the 5' end of the AOX1 3' termination sequence and the 3' end of the HSA structural gene.

A.. Creation of pXXHSA113 Δ Clal

1 μ g of pXHSA113 Δ Clal was digested for 4 hours at 37 °C with 10 units of EcoRI in 100 μ l REact 3 buffer. The digestion mixture was phenol extracted and DNA precipitated as detailed in Example VI. DNA precipitate was dissolved in 20 μ l water and digested for 1 hour at 37 °C with 20 units of Bsu36I in 100 μ l of Bsu36I buffer. The digestion mixture was phenol extracted, DNA precipitated and dissolved in 100 μ l of water as detailed in Example VI. Approximately 100 ng of EcoRI and Bsu36I-cleaved DNA was mixed with 10 ng of annealed oligonucleotides 5'-TTAGGCTTATAAG (SEQ ID NO:10) and 5'-AATTCTTATAAGCC (SEQ ID NO:11) and ligated overnight at 4 °C in 20 μ l of T₄ DNA ligase buffer containing 100 μ g/ml BSA and 10 units of T₄ DNA ligase. The ligation mixture was used to transform E. coli to obtain pXXHSA113 Δ Clal. In this plasmid the sequence between Bsu36I and EcoRI (SEQ ID NO:12) present in pXHSA113 Δ Clal shown below:

Bsu36I

5' CCTTAGGCTTATAACATCTCTACATTTAAAGCATCTCAGCCTACCATGAGAATAAGAGAAAGAAAATGAAGATCA
AAAGCTTATTCATCTGTGTTTCTTTTCGTTGGTGTAAGCCACACCCCTGTCTAAAAACATAAATTCTTTAATC
ATTTTGCCCTCTTTTCTCTGTGCTTCAATTAATAAAAAATGGAAAGAATCTAAAAAAAAAAAAAAAAAGGAATTC
EcoRI

is replaced by 5' CC TTA GGC TTA TAA GAATTC (SEQ ID NO:13)

Bsu36I

EcoRI

B. Creation of pHSA413

1 μ g of pXXHSA113 Δ Clal was digested for 4 hours at 37 °C with Clal in 100 μ l of REact 1 buffer. Following digestion the reaction mixture was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in 200 μ l reaction volume for 30 minutes at 37 °C. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated and dissolved in

water at a concentration of approximately 10 ng/μl as described in step A and stored at -20° C.

Approximately 100 ng (10 μl) of ClaI cleaved-phosphatased pXXHSA113ΔCla was mixed with approximately 80 ng (4 μl) of PstI digested phosphatased and ClaI-cleaved pA0807N (see paragraph 2 in step 3 of Example VI), 4 μl of 5X ligase buffer, 2 μl of 1 mg/ml BSA and ligated overnight at 4° C using 1 unit of T₄ DNA ligase. The ligation mixture was used to transform *E. coli* DG75' to obtain pHSA413. The pHSA413 plasmid from this ligation contains the complete pXXHSA113ΔCla sequence linked to the HIS4 gene and the AOXI 3' second insertable sequence derived from pA0807N. The relative orientation of the components of the pHSA413 plasmid is the same as that shown in Figure 7 for plasmid pHSA113.

10 Example VIII

Transformation of *Pichia pastoris* with pHSA313 and pHSA413

A. Vector preparation

15 About 10 μg each of pHSA313, pHSA413, and pA0807N (negative control) were digested for 12 hours at 37° C in 200 μl of HS buffer with 50 units of NotI. The digested DNA samples were phenol extracted, precipitated as described in Example VI, dissolved in 20 μl of CaS, and were then used for transformation of *Pichia pastoris* GS115. About 10 μg each of pHSA313, pHSA413, and pA0807N were also digested with 20 units of SstI for 12 hours at 37° C in 200 μl of REact 2 buffer containing 100 μg/ml of BSA. The digested DNA samples were extracted with phenol, precipitated as described in Example VI and dissolved in 20 μl of CaS.

B. Cell Growth

25 *Pichia pastoris* GS115 (NRRL Y-15851) was inoculated into about 10 ml of YPD medium and shake cultured at 30° C for 12-20 hours. 100 ml of YPD medium was inoculated with a seed culture to give an OD₆₀₀ of about 0.001. The medium was cultured in a shake flask at 30° C for about 12-20 hours. The culture was harvested when the OD₆₀₀ was about 0.2-0.3 by centrifugation at 1555 g for 5 minutes using a Sorvall RB5C.

C. Preparation of Spheroplasts

35 The cells were washed in 10 ml of sterile water, and then centrifuged at 1500 g for 5 minutes. (Centrifugation is performed after each cell wash at 1500 g for 5 minutes using a Sorvall RT6000B unless otherwise indicated.) The cells were washed once in 10 ml of freshly prepared SED, once in 10 ml of sterile 1M sorbitol, and finally resuspended in 10 ml of SCE buffer. 7.5 μl of 3 mg/ml Zymolyase (100,000 units/g, obtained from Miles Laboratories) was added to the cell suspension. The cells were incubated at 30° C for about 10 minutes. (A reduction of 60% in OD₆₀₀ in 5% SDS can be utilized as a correct time marker.) The spheroplasts were washed in 10 ml of sterile 1 M sorbitol by centrifugation at 700 g for 5-10 minutes. 10 ml of sterile CaS was used as a final cell wash, and the cells were centrifuged again at 700 g for 5-10 minutes and then resuspended in 0.6 ml of CaS.

D. Transformation

45 *Pichia pastoris* GS115 cells were transformed with 10 μg of linearized DNA (see step A) using the spheroplast transformation technique of Sreekrishna et al, Gene 59, 115-125 (1987). DNA samples were added (up to 20 μl volume) to 12 x 75 mm sterile polypropylene tubes. (DNA should be in a suitable buffer such as TE buffer or CaS.) 100 μl of spheroplasts were added to each DNA sample and incubated at room temperature for about 20 minutes. 1 ml of PEG solution was added to each sample and incubated at room temperature for about 15 minutes and centrifuged at 700 g for 5-10 minutes. SOS (150 μl) was added to the pellet and incubated for 30 minutes at room temperature. Finally 850 μl of 1M sorbitol was added.

E. Regeneration of Spheroplasts

55 A bottom agarose layer of 20 ml of regeneration agar SDR was poured per plate at least 30 minutes before transformation samples were ready. In addition, 8 ml aliquots of regeneration agar were distributed to 15 ml conical bottom Corning tubes in a 45° C water bath during the period that transformation samples

were in SOS. Aliquots of 50 or 250 μ l of the transformed sample was added to the 8 ml aliquots of molten regeneration agar held at 45 °C and poured onto plates containing the solid 20 ml bottom agar layer. The plates were incubated at 30 °C for 3-5 days.

5 F. Selection of Transformants

Transformants were selected for by culturing on SDR, a media lacking histidine. The colonies which grew in the absence of histidine were also screened for "methanol-slow" phenotype, indicating displacement of the AOX1 structural gene by the NotI DNA fragment) in the case of transformants obtained using
10 NotI linearized vectors. Several transformed GS115 cells showing "methanol-normal" (those obtained with SstI linearized DNA) and methanol-slow were then cultured and assayed for the production of HSA.

Example IX

15 Methanol induced secretion of HSA in GS115/pHSA313, and GS115/pHSA413 Integrative Transformants

Pichia pastoris GS115 strains transformed with pHSA313 and pHSA413 were analysed for HSA secretion in shake tube cultures. Both methanol-slow and methanol-normal strains were used. In each case 36 independent clones were studied. Transformants obtained with pA0807N served as negative controls. A
20 protocol was developed to ensure efficient secretion and stable accumulation of HSA in the culture medium.

Cells were grown to saturation in 10 ml BMGR or BMGY, and were placed in 50 ml tubes (2-3 days). The cells would be in the range of 10-20 A_{600} units. The cells were harvested, the supernatant liquid was discarded, and then the pellet was resuspended in 2 ml of BMMR or BMMY. The tube was covered with a sterile gauze (cheese cloth) instead of a cap. The tube(s) were then returned to a 30 °C shaker. At the end
25 of 2-3 days, the cells were pelleted, and the supernatant assayed for product. The pellets could be resuspended with fresh medium and returned to the shaker for renewed secretion. With *Pichia*-HSA strains, 10 μ l of media supernatant was sufficient for analysis by SDS-PAGE followed by Coomassie staining. Under these conditions a single band of 67 kD corresponding to HSA was observed. There was no significant difference between the expression levels of GS115/pHSA313 vs GS115/pHSA413 transformants, suggesting
30 that deleting the 3' untranslated sequences from the HSA gene present in pHSA313 did not significantly affect expression levels. No significant difference in the HSA expression level was observed between methanol-slow vs methanol-normal transformants, in shake flask cultures. This suggests that disruption of AOX1 was not essential for efficient HSA expression. As expected, HSA was absent in both the culture medium and the cell extract of GS115/pA0807N transformants (negative control).

35

Example X

Batch-Fed Fermentation of Mut⁻ *Pichia pastoris* for Production of HSA

Pichia pastoris GS115:pHSA 413-6 was inoculated into a 20 liter BiolaFitte fermentor with an 8.5 l working volume. The inoculum was prepared in the following manner: a culture was grown on a YM plate and then transferred to 100 ml YM broth in a shake flask and grown for about 24 hours. 50 mls of this culture was transferred to 1 liter of YM broth in a shake flask and also grown for about 24 hours. 1 liter of this was then transferred to 8.5 liters of fermentor medium in the BiolaFitte fermentor. Fermentor medium
45 consisted of Minimal salts + biotin + 5 percent glycerol. Batch growth conditions included the following: pH = 5.8 (controlled with NH₃), temperature = 30 °C, and percent dissolved oxygen greater than 20 percent air saturation.

Glycerol exhaustion was complete after about 24 hours, at which time a slow methanol feed was begun at a rate of 10-15 ml/hr. The methanol concentration was monitored in the fermentor and the feed rate was
50 adjusted to maintain a concentration of 0.5-0.9 percent of methanol in the broth.

Secreted HSA in the media was measured quantitatively by desitometry of Coomassie blue stained polyacrylamide gels containing SDS (SDS-PAGE). Areas were referenced to a series of known weights of authentic HSA run on the same SDS-PAGE gels. The data from these gels is included in Table III.

55

Table III

Production of HSA by Batch-Fed Fermentation					
Run	Strain	Run pH	Hrs. MeOH	Dry Cell Wt.	HSA in Broth g/l
1	GS115:pHSA 413-6	5.79	101	ND	2.13
2	GS115:pHSA 413-6	5.85	237	101	3.39
3	GS115:pHSA 413-6	5.85	265	98.12	2.70
4	GS115:pHSA 413-6	5.97	258	117	2.90

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kotikanyadan Sreekrishna et al.

(ii) TITLE OF INVENTION: Expression of Human Serum Albumin in
Pichia pastoris

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:

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(D) STATE: OK.

(E) COUNTRY: USA

(F) ZIP: 74005

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM PC

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: Display Write 4

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Hal Brent Woodrow

(B) REGISTRATION NUMBER: 32,501

(C) REFERENCE/DOCKET NUMBER: 32747

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 1-918-661-0624

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 940 bp
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

15 (ix) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	AGATCTAACA	TCCAAAGACG	AAAGGTTGAA	TGAAACCTTT	TTGCCATCCG	ACATCCACAG	60
20	GTCCATTCTC	ACACATAAGT	GCCAAACGCA	ACAGGAGGGG	ATACACTAGC	AGCAGACCGT	120
	TGCAAACGCA	GGACCTCCAC	TCCTCTTCTC	CTCAACACCC	ACTTTTGCCA	TCGAAAAACC	180
	AGCCCAGTTA	TTGGGCTTGA	TTGGAGCTCG	CTCATTCCAA	TTCCTTCTAT	TAGGCTACTA	240
25	ACACCATGAC	TTTATTAGCC	TGTCTATCCT	GGCCCCCCTG	GCGAGGTTCA	TGTTTGTTTA	300
	TTTCCGAATG	CAACAAGCTC	CGCATTACAC	CCGAACATCA	CTCCAGATGA	GGGCTTTCTG	360
	AGTGTGGGGT	CAAATAGTTT	CATGTTCCCC	AAATGGCCCA	AAACTGACAG	TTTAAACGCT	420
30	GTCTTGGAAC	CTAATATGAC	AAAAGCGTGA	TCTCATCCAA	GATGAACTAA	GTTTGGTTCG	480
	TTGAAATGCT	AACGGCCAGT	TGGTCAAAAA	GAAACTTCCA	AAAGTCGGCA	TACCGTTTGT	540
	CTTGTTTGGT	ATTGATTGAC	GAATGCTCAA	AAATAATCTC	ATTAATGCTT	AGCGCAGTCT	600
35	CTCTATCGCT	TCTGAACCCC	GGTGCACCTG	TGCCGAAACG	CAAATGGGGA	AACACCCGCT	660
	TTTTGGATGA	TTATGCATTG	TCTCCACATT	GTATGCTTCC	AAGATTCTGG	TGGGAATACT	720
	GCTGATAGCC	TAACGTTTAT	GATCAAAATT	TAAGTGTCTT	AACCCCTACT	TGACAGCAAT	780
40	ATATAAACAG	AAGGAAGCTG	CCCTGTCTTA	AACCTTTTTT	TTTATCATCA	TTATTAGCTT	840
	ACTTTCATAA	TTGCGACTGG	TTCCAATTGA	CAAGCTTTTG	ATTTTAACGA	CTTTTAACGA	900
45	CAACTTGAGA	AGATCAAAAA	ACAATAATT	ATTCGAAACG			940

50

65

(3) INFORMATION FOR SEQ ID NO:2:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 600 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: Genomic DNA

(1x) SEQUENCE DESCRIPTION: SEQ ID NO:2:

5	AAAGTAAACC	CCATTCAATG	TTCGAGATT	TAGTATACTT	GCCCCTATAA	GAAACGAAGG	60
15	ATTCAGCTT	CCTACCCCA	TGAACAGAAA	TCTTCCATTT	ACCCCCCACT	GGAGAGATCC	120
	GCCCAAACGA	ACAGATAATA	GAAAAAAGAA	ATTCGGACAA	ATAGAACACT	TTCTCAGCCA	180
	ATTAAAGTCA	TTCATGCAC	TCCCTTTAGC	TGCCGTTCCA	TCCCTTTGTT	GAGCAACACC	240
20	ATCGTTAGCC	AGTACGAAAG	AGGAAACTTA	ACCGATACCT	TGGAGAAATC	TAAGGCGCGA	300
	ATGAGTTTAG	CCTAGATATC	CTTAGTGAAG	GGTGTTCGA	TACCTTCTCC	ACATTCAGTC	360
	ATAGATGGGC	AGCTTTGTTA	TCATGAAGAG	ACGGAAACGG	GCATTAAAGG	TTAACCGCCA	420
25	AATTATATAA	AAGACAACAT	GTCCCCAGTT	TAAAGTTTTT	CTTTCCTATT	CTTGATCCT	480
	GAGTGACCGT	TGTGTTTAAT	ATAACAAGTT	CGTTTTAACT	TAAGACCAAA	ACCAGTTACA	540
30	ACAAATTATA	ACCCCTCTAA	ACACTAAAGT	TCACTCTTAT	CAAACTATCA	AACATCAAAA	600

(4) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1830 bp
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(11) MOLECULE TYPE: Genomic DNA

(1x) SEQUENCE DESCRIPTION: SEQ ID NO:3:

5	ATG	AAG	TGG	GTA	ACC	TTT	ATT	TCC	CTT	CTT	TTT	CTC	TTT	AGC	TCG	45
	Met	Lys	Trp	Val	Thr	Phe	Ile	Ser	Leu	Leu	Phe	Leu	Phe	Ser	Ser	
		-35					-30					-25				
15	GCT	TAT	TCC	AGG	GGT	GTG	TTT	CGT	CGA	GAT	GCA	CAC	AAG	AGT	GAG	90
	Ala	Tyr	Ser	Arg	Gly	Val	Phe	Arg	Arg	Asp	Ala	His	Lys	Ser	Glu	
		-20					-15					-10				
20	GTT	GCT	CAT	CGG	TTT	AAA	GAT	TTG	GGA	GAA	GAA	AAT	TTC	AAA	GCC	135
	Val	Ala	His	Arg	Phe	Lys	Asp	Leu	Gly	Glu	Glu	Asn	Phe	Lys	Ala	
		-5					1				5					
25	TTG	GTG	TTG	ATT	GCC	TTT	GCT	CAG	TAT	CTT	CAG	CAG	TGT	CCA	TTT	180
	Leu	Val	Leu	Ile	Ala	Phe	Ala	Gln	Tyr	Leu	Gln	Gln	Cys	Pro	Phe	
	10					15					20					
30	GAA	GAT	CAT	GTA	AAA	TTA	GTG	AAT	GAA	GTA	ACT	GAA	TTT	GCA	AAA	225
	Glu	Asp	His	Val	Lys	Leu	Val	Asn	Glu	Val	Thr	Glu	Phe	Ala	Lys	
	25					30					35					
35	ACA	TGT	GTT	GCT	GAT	GAG	TCA	GCT	GAA	AAT	TGT	GAC	AAA	TCA	CTT	270
	Thr	Cys	Val	Ala	Asp	Glu	Ser	Ala	Glu	Asn	Cys	Asp	Lys	Ser	Lue	
	40					45					50					
40	CAT	ACC	CTT	TTT	GGA	GAC	AAA	TTA	TGC	ACA	GTT	GCA	ACT	CTT	CGT	315
	His	Thr	Leu	Phe	Gly	Asp	Lys	Leu	Cys	Thr	Val	Ala	Thr	Leu	Arg	
	55					60					65					
45	GAA	ACC	TAT	GGT	GAA	ATG	GCT	GAC	TGC	TGT	GCA	AAA	CAA	GAA	CCT	360
	Glu	Thr	Tyr	Gly	Glu	Met	Ala	Asp	Cys	Cys	Ala	Lys	Gln	Glu	Pro	
	70					75					80					
50	GAG	AGA	AAT	GAA	TGC	TTC	TTG	CAA	CAC	AAA	GAT	GAC	AAC	CCA	AAC	405
	Glu	Arg	Asn	Glu	Cys	Phe	Leu	Gln	His	Lys	Asp	Asp	Asn	Pro	Asn	
	85					90					95					
55	CTC	CCC	CGA	TTG	GTG	AGA	CCA	GAG	GTT	GAT	GTG	ATG	TGC	ACT	GCT	450
	Leu	Pro	Arg	Leu	Val	Arg	Pro	Glu	Val	Asp	Val	Met	Cys	Thr	Ala	
	100					105					110					
60	TTT	CAT	GAC	AAT	GAA	GAG	ACA	TTT	TTG	AAA	AAA	TAC	TTA	TAT	GAA	495
	Phe	His	Asp	Asn	Glu	Glu	Thr	Phe	Leu	Lys	Lys	Tyr	Leu	Tyr	Glu	
	115					120					125					

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5	ATT Ile 130	GCC Ala	AGA Arg	AGA Arg	CAT His	CCT Pro 135	TAC Tyr	TTT Phe	TAT Tyr	GCC Ala	CCG Pro	GAA Glu	CTC Leu	CTT Leu	TTC Phe	540
	TTT Phe 145	GCT Ala	AAA Lys	AGG Arg	TAT Tyr	AAA Lys 150	GCT Ala	GCT Ala	TTT Phe	ACA Thr	GAA Glu	TGT Cys	TGC Cys	CAA Gln	GCT Ala	585
10	GCT Ala 160	GAT Asp	AAA Lys	GCT Ala	GCC Ala	TGC Cys 165	CTG Leu	TTG Leu	CCA Pro	AAG Lys	CTC Leu	GAT Asp	GAA Glu	CTT Leu	CGG Arg	630
15	GAT Asp 175	GAA Glu	GGG Gly	AAG Lys	GTT Val	TCG Ser 180	TCT Ser	GCC Ala	AAA Lys	CAG Gln	AGA Arg	CTC Leu	AAG Lys	TGT Cys	GCC Ala	675
	AGT Ser 190	CTC Leu	CAA Gln	AAA Lys	TTT Phe	GGA Gly 195	GAA Glu	AGA Arg	GCT Ala	TTC Phe	AAA Lys	GCA Ala	TGG Trp	GCA Ala	GTA Val	720
20	GCT Ala 205	CGC Arg	CTG Leu	AGC Ser	CAG Gln	AGA Arg 210	TTT Phe	CCC Pro	AAA Lys	GCT Ala	GAG Glu	TTT Phe	GCA Ala	GAA Glu	GTT Val	765
25	TCC Ser 220	AAG Lys	TTA Leu	GTG Val	ACA Thr	GAT Asp 225	CTT Leu	ACC Thr	AAA Lys	GTC Val	CAC His	ACG Thr	GAA Glu	TGC Cys	TGC Cys	810
30	CAT His 235	GGA Gly	GAT Asp	CTG Leu	CTT Leu	GAA Glu 240	TGT Cys	GCT Ala	GAT Asp	GAC Asp	AGG Arg	GCG Ala	GAC Asp	CTT Leu	GCC Ala	855
	AAG Lys 250	TAT Tyr	ATC Ile	TGT Cys	GAA Glu	AAT Asn 255	CAA Gln	GAT Asp	TCG Ser	ATC Ile	TCC Ser	AGT Ser	AAA Lys	CTG Leu	AAG Lys	900
35	GAA Glu 265	TGC Cys	TGT Cys	GAA Glu	AAA Lys	CCT Pro 270	CTG Leu	TTG Leu	GAA Glu	AAA Lys	TCC Ser	CAC His	TGC Cys	ATT Ile	GCC Ala	945
40	GAA Glu 280	GTG Val	GAA Glu	AAT Asn	GAT Asp	GAG Glu 285	ATG Met	CCT Pro	GCT Ala	GAC Asp	TTG Leu	CCT Pro	TCA Ser	TTA Leu	GCT Ala	990
45	GCT Ala 295	GAT Asp	TTT Phe	GTT Val	GAA Glu	AGT Ser 300	AAG Lys	GAT Asp	GTT Val	TGC Cys	AAA Lys	AAC Asn	TAT Tyr	GCT Ala	GAG Glu	1035
	GCA Ala 310	AAG Lys	GAT Asp	GTC Val	TTC Phe	TTG Leu 315	GGC Gly	ATG Met	TTT Phe	TTG Leu	TAT Tyr	GAA Glu	TAT Tyr	GCA Ala	AGA Arg	1080

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	AGG Arg 325	CAT His	CCT Pro	GAT Asp	TAC Tyr	TCT Ser 330	GTC Val	GTG Val	CTG Leu	CTG Leu	CTG Leu	AGA Arg	CTT Leu	GCC Ala	AAG Lys	1125
5	ACA Thr 340	TAT Tyr	GAA Glu	ACC Thr	ACT Thr	CTA Leu 345	GAG Glu	AAG Lys	TGC Cys	TGT Cys	GCC Ala	GCT Ala	GCA Ala	GAT Ala	CCT Asp	1170
10	CAT His 355	GAA Glu	TGC Cys	TAT Tyr	GCC Ala	AAA Lys 360	GTG Val	TTC Phe	GAT Asp	GAA Glu	TTT Phe	AAA Lys	CCT Pro	CTT Leu	GTG Val	1215
15	GAA Glu 370	GAG Glu	CCT Pro	CAG Gln	AAT Asn	TTA Leu 375	ATC Ile	AAA Lys	CAA Gln	AAT Asn	TGT Cys	GAG Glu	CTT Leu	TTT Phe	GAG Glu	1260
20	CAG Gln 385	CTT Leu	GGA Gly	GAG Glu	TAC Tyr	AAA Lys 390	TTC Phe	CAG Gln	AAT Asn	GCG Ala	CTA Leu	TTA Leu	GTT Val	CGT Arg	TAC Tyr	1305
	ACC Thr 400	AAG Lys	AAA Lys	GTA Val	CCC Pro	CAA Gln 405	GTG Val	TCA Ser	ACT Thr	CCA Pro	ACT Thr	CTT Leu	GTA Val	GAG Glu	GTG Val	1350
25	TCA Ser 415	AGA Arg	AAC Asn	CTA Leu	GGA Gly	AAA Lys 420	GTG Val	GGC Gly	AGC Ser	AAA Lys	TGT Cys	TGT Cys	AAA Lys	CAT His	CCT Pro	1395
30	GAA Glu 430	GCA Ala	AAA Lys	AGA Arg	ATG Met	CCC Pro 435	TGT Cys	GCA Ala	GAA Glu	GAC Asp	TAT Tyr	CTA Leu	TCC Ser	GTG Val	GTG Val	1440
35	CTG Leu 445	AAC Asn	CAG Gln	TTA Leu	TGT Cys	GTG Val 450	TTG Leu	CAT His	GAG Glu	AAA Lys	ACG Thr	CCA Pro	GTA Val	AGT Ser	GAC Asp	1485
40	AGA Arg 460	GTC Val	ACC Thr	AAA Lys	TGC Cys	TGC Cys 465	ACA Thr	GAA Glu	TCC Ser	TTG Leu	GTG Val	AAC Asn	AGG Arg	CGA Arg	CCA Pro	1530
	TGC Cys 475	TTT Phe	TCA Ser	GCT Ala	CTG Leu	GAA Glu 480	GTG Val	GAT Asp	GAA Glu	ACA Thr	TAC Tyr	GTT Val	CCC Pro	AAA Lys	GAG Glu	1575
45	TTT Phe 490	AAT Asn	GCT Ala	GAA Glu	ACA Thr	TTC Phe 495	ACC Thr	TTC Phe	CAT His	GCA Ala	GAT Asp	ATA Ile	TGC Cys	ACA Thr	CTT Leu	1620
50	TCT Ser 505	GAG Glu	AAG Lys	GAG Glu	AGA Arg	CAA Gln 510	ATC Ile	AAG Lys	AAA Lys	CAA Gln	ACT Thr	GCA Ala	CTT Leu	GTT Val	GAG Glu	1665

55

	CTT	GTG	AAA	CAC	AAG	CCC	AAG	GCA	ACA	AAA	GAG	CAA	CTG	AAA	GCT	1710
	Leu	Val	Lys	His	Lys	Pro	Lys	Ala	Thr	Lys	Glu	Gln	Leu	Lys	Ala	
	520					525					530					
5	GTT	ATG	GAT	GAT	TTC	GCA	GCT	TTT	GTA	GAG	AAG	TGC	TGC	AAG	GCT	1755
	Val	Met	Asp	Asp	Phe	Ala	Ala	Phe	Val	Glu	Lys	Cys	Cys	Lys	Ala	
	535					540					545					
10	GAC	GAT	AAG	GAG	ACC	TGC	TTT	GCC	GAG	GAG	GGT	AAA	AAA	CTT	GTT	1800
	Asp	Asp	Lys	Glu	Thr	Cys	Phe	Ala	Glu	Glu	Gly	Lys	Lys	Leu	Val	
	550					555					560					
15	GCT	GCA	AGT	CAA	GCT	GCC	TTA	GGC	TTA	TAA						1830
	Ala	Ala	Ser	Gln	Ala	Ala	Leu	Gly	Leu							
	565					570										

(5) INFORMATION FOR SEQ ID NO:4:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: Oligonucleotide

(1x) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCTCACACG CCTTTGAATT C ATG AAG TGG GTA ACC 36

Met Lys Trp Val Thr

1 5

(6) INFORMATION FOR SEQ ID NO:5:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: Oligonucleotide

(1x) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCC TTA GGC TTA TAAGAATTCA GTTTAAAGC ATCTCAG 39

Ala Leu Gly Leu

570

(7) INFORMATION FOR SEQ ID NO:6:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: Screening Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCTGGGAAT CC ATG AAG 18
Met Lys
1

(8) INFORMATION FOR SEQ ID NO:7:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: Screening Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTA TAAGAATTCA GTTA 18
Leu
573

(9) INFORMATION FOR SEQ ID NO:8:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGAAACG ATG AAG TGG 16
Met Lys Trp

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTTACCCACT TCATCGTTT 19

(11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTAGGCTTAT AAG 13

(12) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AATTCTTATA AGCC 14

(13) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 231bp
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Linker Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

CCTTAGGCTT ATAACATCTC TACATTTAAA AGCATCTCAG CCTACCATGA GAATAAGAGA 60
AAGAAAATGA AGATCAAAAG CTTATTCATC TGTGTTTTCT TTTTCGTTGG TGTAAGCCA 120
ACACCCGTGC TAAAAACAT AAATTTCTTT AATCATTTTG CCTCTTTTTC TCTGTGCTTC 180
AATTAATAAA AAATGGAAAG AATCTAAAAA AAAAAAAAAA AAAAGGAATT C 231

```

(14) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20bp
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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CCTTAGGCTT ATAAGAATTC 20

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Claims

1. An expression cassette for the production of HSA in *Pichia pastoris* comprising
 - a) a *Pichia pastoris* 5' regulatory region having a 5' end and a 3' end selected from a *Pichia pastoris* AOX1 5' regulatory region and a *Pichia pastoris* DAS1 5' regulatory region wherein the 3' end of the 5' regulatory region is operably linked to
 - b) a HSA structural gene encoding a HSA signal sequence and a mature HSA protein, having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within no more than 11 deoxyribonucleotides of the 5' end of said HSA structural gene; operably linked to
 - c) a suitable 3' termination sequence.
2. The expression cassette of claim 1 wherein the 5' regulatory region is selected from the AOX1 5' regulatory region and the DAS1 5' regulatory region from *Pichia pastoris*.
3. The expression cassette of claim 1 wherein the 3' termination sequence is isolated from a *Pichia pastoris* gene selected from an AOX1 gene, a DAS1 gene, a p40 gene and a HIS4 gene.
4. The expression cassette of claim 1 wherein the expression cassette is incorporated into a vector selected from circular plasmids and linear plasmids, the latter preferably being integrative site-specific vectors.

5. The expression cassette of claim 1 wherein the expression cassette is incorporated into a vector comprising the following serial arrangement:
 - a) a first insertable DNA fragment;
 - b) at least one marker gene; and,
 - 5 c) a second insertable DNA fragment; wherein at least one expression cassette is incorporated either before or after the marker gene of component (b), and the first and second insertable DNA fragments employed are homologous with separate portions of the *Pichia pastoris* genome and the insertable fragments are in the same relative orientation as exist in the *Pichia pastoris* genome.
- 10 6. The expression cassette of claim 5 contained in said vector wherein the first insertable DNA fragment and the second insertable DNA fragment are obtained from the DNA sequences of a gene isolated from *Pichia pastoris* selected from an AOX1 gene, a p40 gene, a DAS1 gene and an HIS4 gene.
- 15 7. The expression cassette of claim 5 contained in said vector wherein the marker gene is selected from a *Pichia pastoris* HIS4 gene, a *Pichia pastoris* ARG4 gene, a *Saccharomyces cerevisiae* SUC2 gene, a G418^R gene of bacterial transposon Tn601 and a G418^R gene of bacterial transposon Tn903.
8. The expression cassette of claim 5 wherein said vector comprises
 - a) a first insertable DNA fragment which is an operable 5' regulatory region from the AOX1 gene being about one kilobase in length isolated from *Pichia pastoris* operably linked to
 - 20 b) a HSA structural gene encoding a HSA signal sequence and a mature HSA protein having a 5' end and a 3' end wherein the HSA structural gene has a ATG start codon within no more than 8 deoxyribonucleotides of the 5' end of said HSA structural gene; operably linked to
 - c) the 3' termination sequence of the AOX1 gene isolated from *Pichia pastoris*; operably linked to
 - 25 d) a marker gene which is the HIS4 gene isolated from *Pichia pastoris*; operably linked to
 - e) a second insertable DNA fragment which is about 0.65 kilobases of the AOX1 3' termination sequence.
9. The expression cassette of claim 8 wherein the HSA structural gene has a ATG start codon with either the deoxyribonucleotide AGGAATTC or no deoxyribonucleotide 5' of said ATG start codon.
- 30 10. A *Pichia pastoris* cell transformed with an expression cassette according to any of claims 1 to 9.
11. The *Pichia pastoris* cell of claim 10 wherein the *Pichia pastoris* cell to be transformed is selected from *Pichia pastoris* GS115 (NRRL Y-15851), *Pichia pastoris* GS190 (NRRL Y-18014), *Pichia pastoris* PPF1 (NRRL Y-18017), *Pichia pastoris* (NRRL Y-11430) and *Pichia pastoris* (NRRL Y-11431), wherein *Pichia pastoris* GS 115 (NRRL Y-15851) is the most preferred *Pichia pastoris* cell.
- 35 12. A process for the secretion of HSA from transformed *Pichia pastoris* cells according to claim 10 or 11 comprising
 - 40 a) transforming a *Pichia pastoris* cell with at least one vector having at least one expression cassette according to any of claims 1 to 9, and
 - b) culturing the resulting transformed *Pichia pastoris* cell under suitable conditions to obtain the production of HSA.
- 45
- 50
- 55

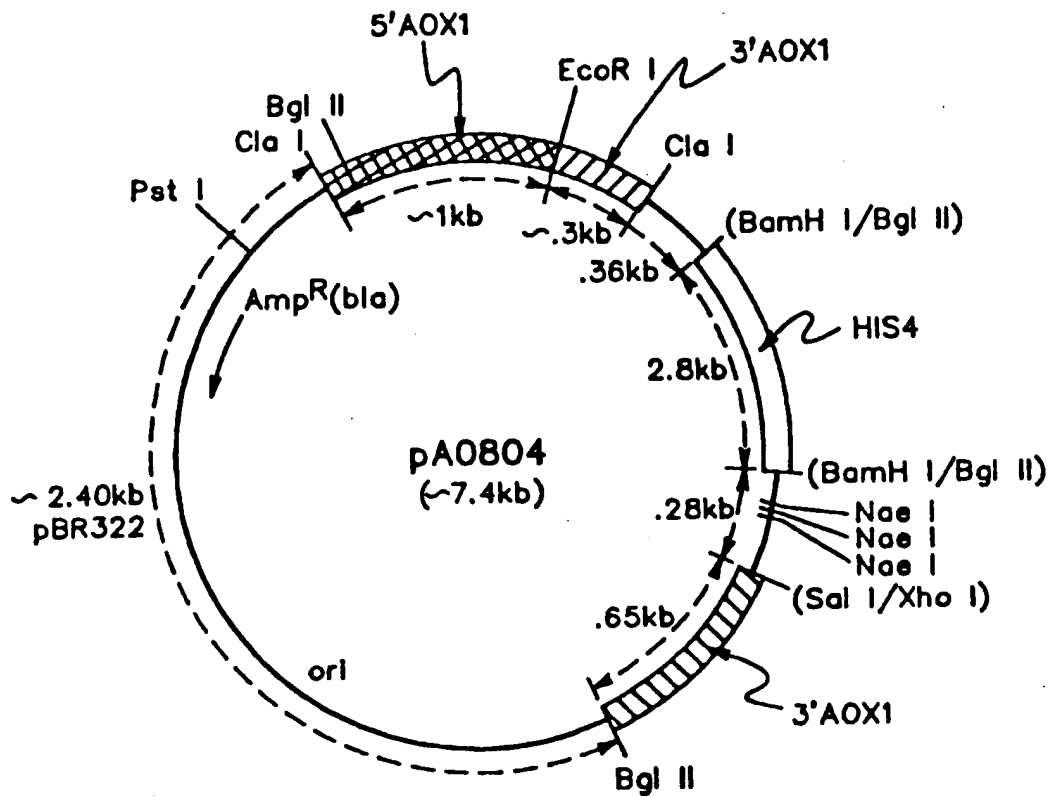


FIG. 1

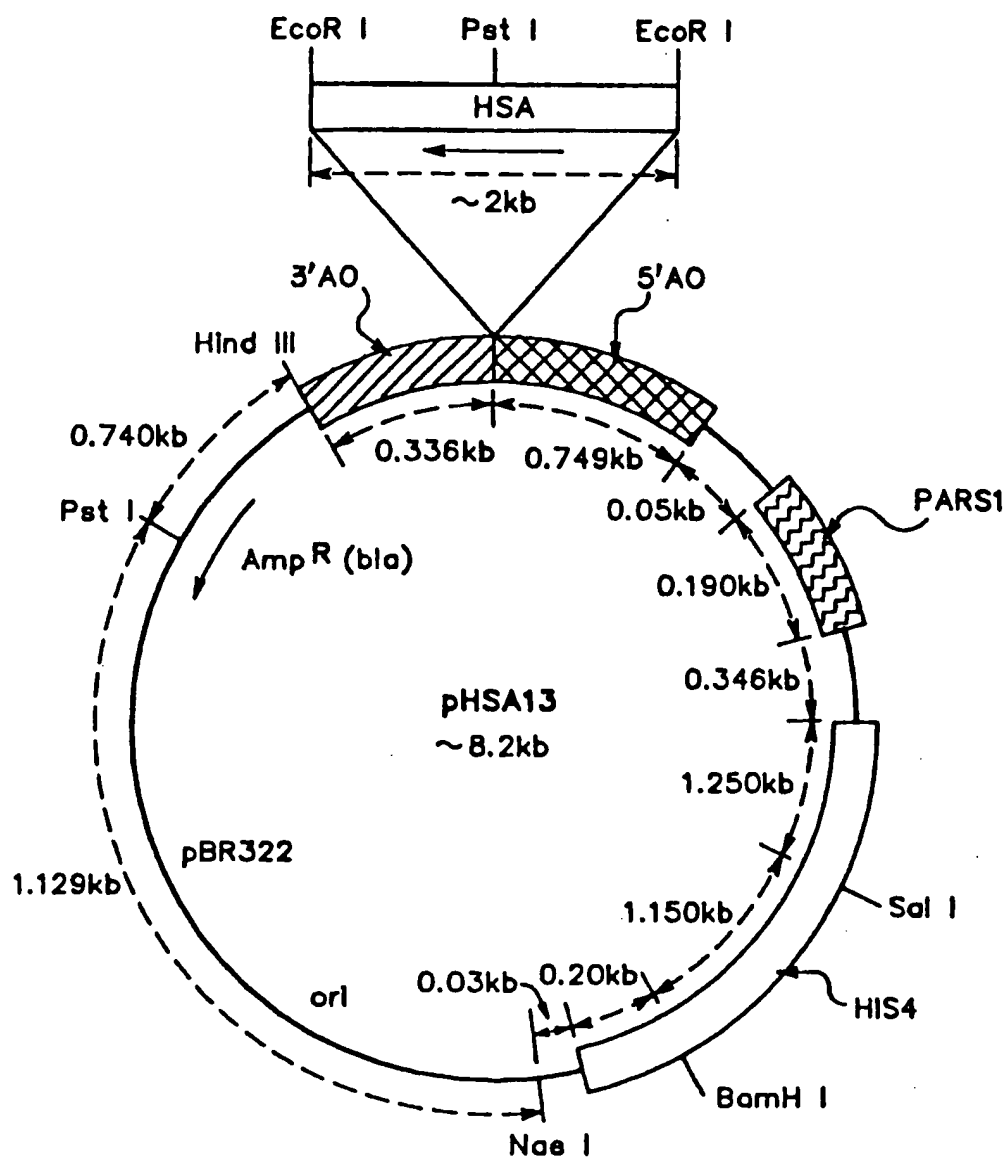


FIG. 2

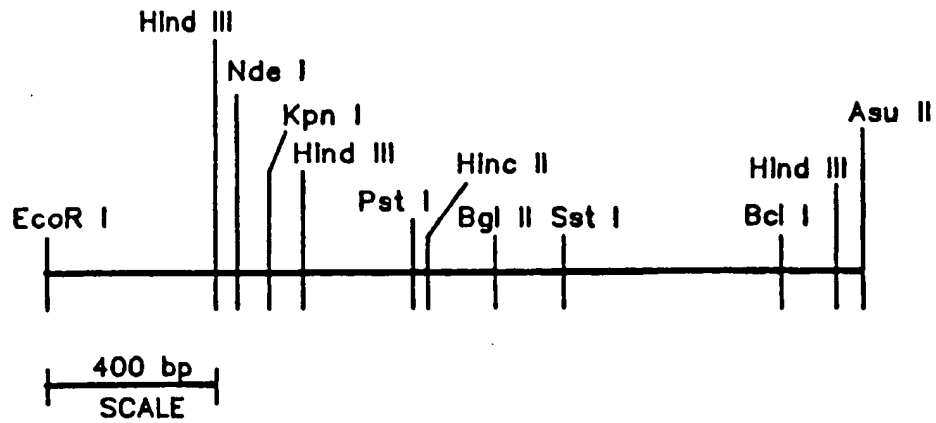


FIG. 3

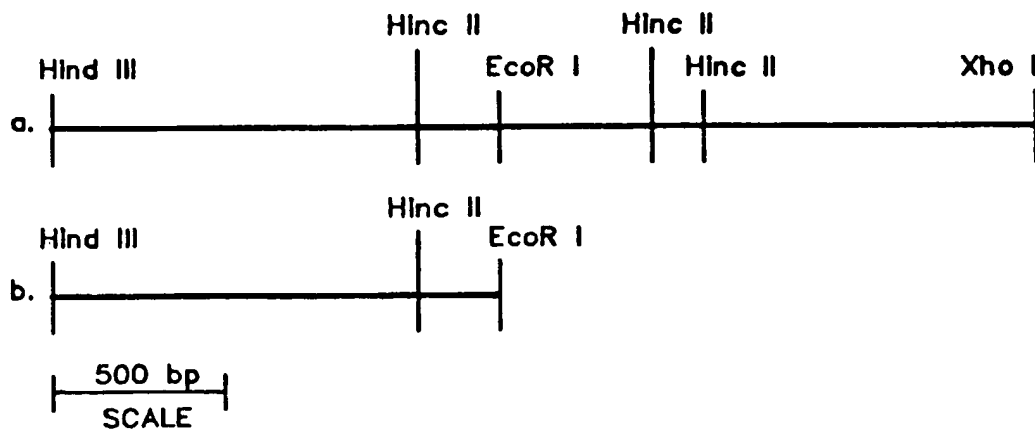


FIG. 4

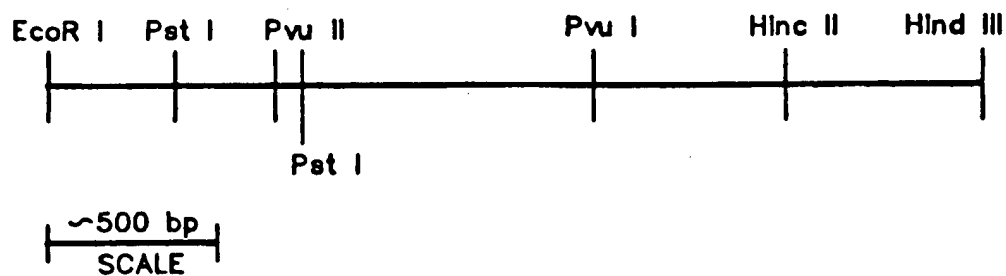


FIG. 5

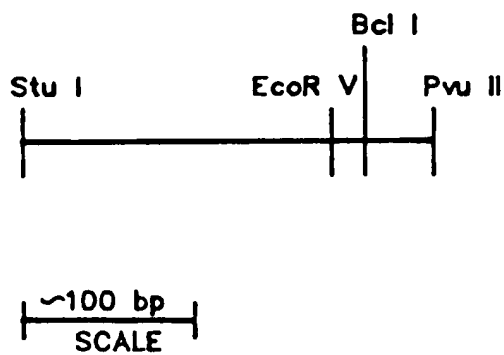


FIG. 6

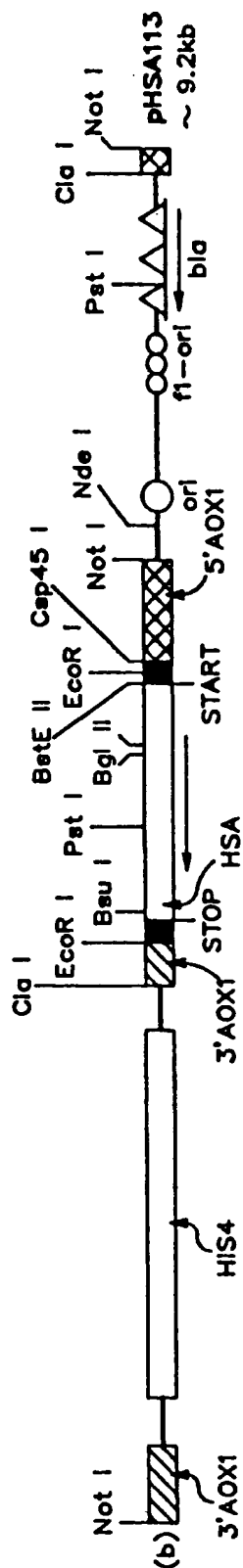


FIG. 7

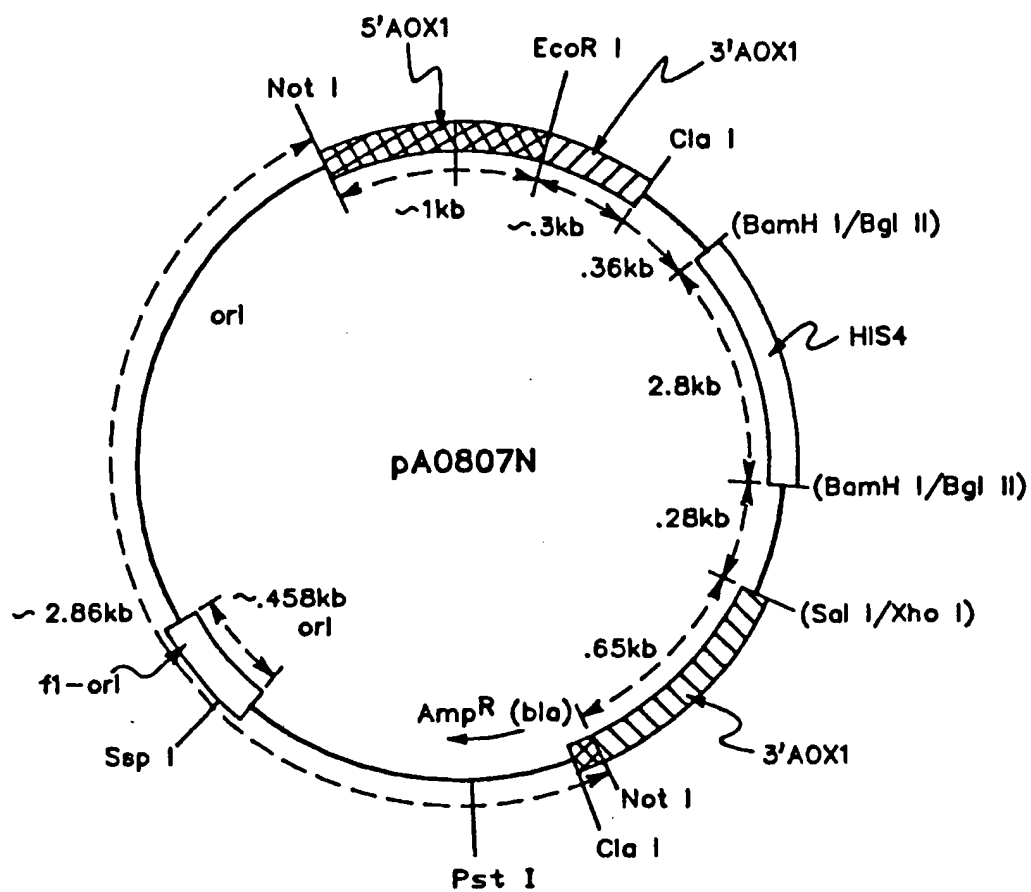


FIG. 8